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Reference materials — Examples of reference materials for qualitative properties

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TECHNICAL REPORT

ISO/TR 79

First edition
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Reference materials - Examples of reference materials for qualitative properties

Matériaux de référence - Exemples de matériaux de référence pour les propriétés qualitatives

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Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
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Foreword

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The committee responsible for this document is ISO/REMCO, *Committee on reference materials*.

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Introduction

In 2007, ISO/REMCO created an ad hoc group (AHG) to investigate the need for guidance on the production of Reference Material (RM) certified for a qualitative property. AHG 01 carried out a gap analysis, contacting 12 organizations and bodies using qualitative RMs and reviewed 13 documents referring to qualitative RMs. Based on this gap analysis, ISO/REMCO decided in 2008 to create a working group (WG) and to entrust it with the drafting of an ISO document.

Due to the limited information submitted in the following years to WG13, the drafting of internationally harmonized guidance turned out to be impossible. Instead, it was decided to focus on an ISO Technical Report (TR), which summarizes the state of the art of the production of qualitative RMs. This TR lists examples of RMs which are either certified for a qualitative property or which can be considered as in-house RMs characterized for a qualitative property. Therefore, many of the RM examples listed here are based on the principles elaborated in ISO Guide 35[1] and ISO Guide 80.[2] The examples represent the experience gathered by various organizations and bodies and their interpretation of qualitative properties, but did not undergo a consensus building process.

In this TR, the following six RM examples are presented:

a) the certification of RMs for their DNA sequences by an ISO Guide 34[3] accredited reference material producer (RMP) (Clause 2);

b) the in-house characterization of organic chemicals as RMs for identification purposes by a laboratory (Clause 3);

c) the identification of a RM biospecimen by an ISO/IEC 17020[4] accredited tissue bank (Clause 4);

d) the development of a reference material for darnel seed identity (Clause 5);

e) the classification and between-sample homogeneity testing of a freshwater cultured pearl (Clause 6);

f) European Pharmacopoeia reference standards for qualitative analysis (Clause 7).

The lack of international standardization in the area of qualitative properties has been recognized by several groups. This includes WG 2 of the Joint Committee for Guides in Metrology (JCGM), officially responsible for the International Vocabulary of Metrology (VIM),[5] which investigates updating and expanding the VIM to cover also qualitative properties. As these discussions are on-going, the terminology used in the various examples presented in this TR may differ, e.g. some groups refer to qualitative properties as nominal properties. Likewise, no agreement has yet been made on international level if the term measurement is limited to quantitative properties or may as well be used for qualitative properties. To foster the readability of this TR, the term *qualitative property* has been given preference and the term *measurement* has been restricted to its use in conjunction with quantitative properties, following the recommendations expressed by the majority of ISO/REMCO delegates during their 37th annual meeting in 2014.

Due to the lack of common guidance on the production of RMs for qualitative properties, the approaches and understanding of terms properly defined for quantitative properties (e.g. homogeneity and traceability) are differently interpreted and applied for qualitative properties by the various organizations and bodies which contributed to this collection of examples. Likewise, the border between qualitative and quantitative properties is differently interpreted. Ordinal properties are perceived by some groups to be restricted to quantitative properties, while others suggest distinguishing between quantitative and qualitative order.

As the predominant aim of this TR is to contribute to the on-going discussion, these differences were on purpose maintained.

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During the writing of ISO/TR 79, ISO/REMCO WG 13 identified a number of discussion items, which could not yet be answered with consensus, but which are considered to be crucial in case further efforts will be made to transform this TR into a Guide.

— The expression of confidence related to identification is discussed and in the majority of the cases, no uncertainty is estimated, although experts agree that the probability for a wrong identification forms also part of the result. The identity of an object does not have an uncertainty; however, the assessment of the identity of an object is related to the possibility for misclassification. Ways to estimate the uncertainty of qualitative analysis are especially suggested in the area of DNA sequencing.[6] At the same time, several areas require an assessment uncertainty equaling zero, like e.g. the classification of blood group values.[7]

— Forward/backward DNA sequencing is considered by many experts as an orthogonal method or method free of parameters influencing the result. At the same time, the question is asked what makes DNA sequencing specific.

— Heterogeneity of materials used as RM for qualitative property identification does not necessarily ruin the intended use. Ways are needed to check to which extend for instance inhomogeneity can be accepted.

— A working group at AOAC International developed internationally harmonized guidelines for the validation of qualitative binary chemistry methods.[8] The Guidelines for Validation of Qualitative Binary Chemistry Methods approach the question from the view point of the method. The question whether the RM used in presence/absence testing needs to be certified for a quantitative or qualitative property has not been discussed in this working group so far.

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Reference materials - Examples of reference materials for qualitative properties

1 Scope

This Technical Report summarizes the state of the art of the production and certification or characterization of qualitative property reference materials (RMs).

The need for guidance documents for the production of RMs certified for qualitative properties was recognized by many experts. At the same time, the available information was found to be too immature to develop an internationally accepted guidance document. Additionally, the lack of an international vocabulary for terms and definitions for qualitative properties made it more difficult for the experts from various testing areas to communicate with each other.

ISO/TR 79 summarizes the available expertise. It aims to contribute to the on-going discussion on nominal properties and the production of such RMs. The investigation of nominal properties is referred to differently in various specialized areas (examination, classification, identification, testing, observation, etc.). ISO/TR 79 tries to foster the future development of an internationally harmonized guidance document.

2 Reference materials certified for their DNA sequence

2.1 General

The following is a compilation of the certification approaches applied for three reference materials which were certified for their DNA sequence by the Joint Research Centre of the European Commission, Institute for Reference Materials and Reference Materials (IRMM, Geel, BE).

2.2 Selected examples of certified reference materials

CRM ERM-AD427[9] is composed of plasmid DNA certified to contain certain DNA fragments. It is used for the quantification of Genetically Modified Organisms (GMOs) and the calibration of a defined quantitative Polymerase Chain Reaction (PCR) method. The Certified Reference Material (CRM) contains a plasmid carrying two defined 2'-deoxyribonucleic acid (DNA) fragments. The plasmid calibrant is certified by DNA sequencing for containing two specific DNA targets per plasmid. The number ratio between the two targets is equal to 1, allowing the use as calibrant for relative real-time PCR measurements. The DNA sequence identity has been confirmed by dye terminator cycle sequencing with a negligible error probability for the sequence identification.

CRM IRMM-448[10] is composed of genomic DNA extracted from a microorganism and certified for its DNA identity (with the PCR region of interest verified by DNA sequencing). IRMM-448 is used as positive control in a defined qualitative PCR method for food testing. The CRM consists of a purified and freeze-dried genomic DNA (gDNA) of *Campylobacter jejuni* (NCTC11351). The identity of the gDNA was confirmed by DNA sequence analysis of the ceuE gene, supporting the harmonization and validation of PCR methods by their use as taxonomic controls in PCR reactions. An indicative value for the mass of freeze-dried gDNA is given.

CRM IRMM/IFCC-490[11] is composed of plasmid DNA certified for its DNA sequence (whole sequence). IRMM/IFCC-490 is intended to be used as positive control in quantitative PCR in the area of genetic testing. The CRM consists of purified plasmid DNA (pDNA) pUC18 containing a specific fragment of the human Factor II (prothrombin) gene sequence. It is intended to support the validation and the harmonization of PCR-based methods used for the detection of the mutation in the human prothrombin gene. In all cases, PCR amplification is followed either by restriction enzyme digestion, hybridization

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protocols, single-strand conformation polymorphism analysis, melting curve analysis, denaturating gradient gel analysis or sequencing.

2.3 Certification approaches applied

2.3.1 Processing of the materials

2.3.1.1 Processing can be carried out fulfilling the requirements laid down in ISO Guide 34.[3] Detailed knowledge and understanding of the individual processing steps are for the production of qualitative RMs of outmost importance as the knowledge about the raw material used is one way to ensure the desired identity. Likewise, meaningful processing controls are important.

An example is the use of extracted genomic DNA for the RM preparation. Genomic DNA from the desired microorganism strain can be obtained from culture collection centres together with a certificate of analysis confirming the identity of the material. The issuing of the certificate for genomic DNA of a particular microorganism strain is typically based on biochemical-, morphological- and microbiological information. Likewise, this approach of controlled origin can be applied for DNA fragments which will be selected for cloning.

2.3.1.2 Verification of the desired DNA sequence of fragments includes techniques such as:

a) purification of DNA fragment prior to ligation of the amplification product with e.g. PCR fragment purification kit;

b) control of the correct length of the DNA amplicons by agarose gel electrophoresis by comparison to a DNA molecular mass ladder;

c) melting temperature investigation of the qPCR products obtained with defined primers and probes to further confirm the identity of the DNA sequence;

d) assessment of the DNA sequence identity by DNA sequence analysis of the fragment inserted into the vector.

2.3.1.3 For cloning, the conditions should be selected in such a way that the cloning of other fragments than the desired one is prohibited. The following should be considered.

a) The synthetic vectors used for cloning should be a high copy vector from the same incompatibility group. As a result, the transformed bacterial clones can only bear one single plasmid and not a mixture of different plasmids.

b) By repeated plating, it should be ensured that only a single colony is cultivated.

2.3.1.4 The outcome of cloning can be checked by:

a) endonuclease restriction analysis to control correct cloning and to check the orientation of the DNA insert;

b) testing of the resulting plasmid by qualitative PCR for the presence of the insert;

c) assessment of the DNA sequence identity by DNA sequence analysis of either the whole DNA sequence or the DNA fragment of interest.

2.3.1.5 In the case of different DNA fragments inserted into a plasmid in a desired ratio, methods should be employed to verify this ratio.

a) Digital PCR experiments can confirm the expected ratio between two target sequences.

b) Real-time PCR method targeting one of the fragments and using the second fragment as normalizer can be applied provided that amplification efficiencies are equivalent.

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2.3.1.6 The DNA sequence identity needs to be confirmed. This confirmation can either concern the whole DNA sequence (e.g. in case of a plasmid) or one or more parts of the DNA (e.g. the PCR target region in genomic DNA from microorganisms used for identification of strains or different DNA fragments inserted into a plasmid in a desired ratio).

Part of the identity confirmation is carried out during the processing (2.3.1 *Processing of the materials*). Depending on the processing steps, repeated application of confirmation method should be considered (e.g. DNA sequencing at various crucial processing steps and if possible in the final preparation).

To ensure that DNA sequencing data have a low probability of incorrect reads, forward and backwards sequencing (Sanger-based sequencing) should be applied. The probability for incorrect reads can be estimated according to Reference [6].

2.3.1.7 In any case, the following should be taken into account with respect to homology searches of the found DNA sequence:

a) if published sequence data were judged to be trustworthy, a homology search of the found DNA sequence (either whole DNA sequence or DNA fragment of relevance) should be applied;

b) a comparison of the found DNA sequence with e.g. data provided by a culture collection centre should be made.

2.3.2 Purity assessment

2.3.2.1 First step

As a first step in the DNA purity investigation, a list of potential DNA contaminants is made. Their impact on the later use of the CRM is evaluated and ways to check for impacting DNA contaminants are elaborated.

2.3.2.2 Plasmid-based CRMs

2.3.2.2.1 Remaining traces of genomic DNA from host bacterial cell or traces of RNA molecules:

It should be checked if such traces would affect the identity of the plasmid and its suitability for real-time PCR measurements, for instance if a high homology is given. If the sequence identity of the genomic DNA of the bacteria and the DNA fragments of interest is low, it is reasonable to conclude that they would not have an impact. However, as remaining traces may induce a bias in the UV absorbance-based DNA quantification of the plasmid solution, the user should be made aware that this could lead to erroneous estimation of the absolute number of plasmid copies.

2.3.2.2.2 Presence of other plasmids than the one containing the DNA fragment(s) of interest:

a) In order to prevent that other plasmids than the one containing the DNA fragment(s) of interest are present, the synthetic vectors used for cloning should be high copy vectors from the same incompatibility group. As a result, the transformed bacterial clones can only bear one single plasmid. It can therefore be concluded that each single bacterium extracted from one colony contains only one type of plasmid.

b) Presence of other plasmids in higher abundance can be tested by enzymatic restriction, with conditions allowing a full digestion. However, one has to bear in mind that it is very difficult to prove that all plasmids were indeed fully digested, as traces of undigested plasmids will not be visible after gel electrophoresis and ethidium bromide staining.

c) As additional proof of purity, the plasmid DNA can be sequenced completely to verify that the desired sequence was correctly cloned. To confirm the purity, the results should not reveal the presence of a mixed population of plasmids. The sequencing technique should be carefully selected in view of the question to be answered; nowadays, a Next Generation Sequencing (NGS) analysis could be applied, provided that settings are chosen in such way that disturbing impurities are detected.

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d) End-point PCR followed by agarose gel electrophoresis can be used to further investigate the purity. The qualitative PCR should be targeted at the DNA sequence(s) which is (are) considered to be either a problem for the later use of the CRM and/or at the DNA sequence which could be expected as contaminant. However, the ability of this method to investigate the purity of the material is limited and often only a purity of 90 % can be proven. Likewise, digital end-point PCR can be used to check for contamination.

2.3.3 Genomic DNA CRMs

Purity of the gDNA from one cell culture:

a) In order to ensure that the gDNA has the desired identity, the origin of the material should be controlled and documented as well as possible. Genomic DNA from the desired microorganism strain should be obtained from culture collection centres together with a certificate of analysis confirming the identity of the material. The issuing of the certificate for genomic DNA of a particular microorganism strain is typically based on biochemical-, morphological- and microbiological information.

b) As an additional proof of purity, the gDNA could be sequenced. This could be done on the whole genome (if feasible) or limited to part of the sequence of interest (i.e. the target region of a PCR).

2.3.4 Characterization (leading to certified values)

Characterization and value assignment for qualitative DNA CRMs rely to a large extent on proper control of processing and the outcome of the purity assessment. In several cases, the characterization could be classified as confirmation, which confirm that the intended material was processed.

Two possible confirmation techniques are as follows.

a) Control of the restriction pattern of the plasmid DNA samples can be done by gel electrophoresis. Gel electrophoresis can be carried out on the gDNA or on the PCR amplicons generated after performance of a specific PCR.

b) The whole DNA should preferably be sequenced by two independent laboratories/instruments or protocols, using preferably forward and backward sequencing (Sanger-based sequencing). Double stranded DNA should be sequenced on both strands in order to ensure an accurate determination by a twofold-coverage of the generated sequences, as well as a complete characterization of the molecular composition of the double stranded DNA.

In the case that a whole sequencing is not possible or considered not to be of interest, sequencing can e.g. be limited to amplicons generated after performance of a specific PCR. The amplicons need to be purified and cloned into a plasmid, which after purification can be sequenced.

The uncertainty related to the sequence determination by Sanger-based sequencing techniques can often be considered as negligible, as the probability to report a wrong base (error probability) is very low. For further guidance, see Reference [6].

2.3.5 Additional characterization (leading to non-certified values)

In various cases, the DNA amount is useful additional material information. Quantification can be done by fluorometry. However, in the absence of reference materials certified for their DNA amount, the resulting value is an estimation of the DNA quantity.

2.3.6 Suitability study

A suitability study is carried out to ensure that the material is fit for purpose and can be used for its intended use. Intrinsically, the suitability study should be designed in such a way that existing commutability problems would be discovered.

As the examples used in this document concern CRMs certified for their sequence and intended to be used for PCR measurements, the intended use for PCR is elaborated here.

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The correct behaviour of a qualitative CRM for its intended purpose (i.e. as PCR calibrant or positive control in PCR) needs to be verified. Ideally, the performance can be tested by laboratories which are potential (later) users of the CRM.

If the type of DNA from analysed samples and calibrants differ (i.e. gDNA and plasmids), commutability problems should be investigated. The calibrant should behave in the analytical process in the same way as the material under investigation.

Suitability of the CRM might not only depend on certified parameters (DNA sequence) but also on non-certified parameters (i.e. the DNA amount). This should be taken into account in the suitability study as well as in the homogeneity study.

Positive outcome of a suitability study proves that the material is fit for its intended purpose.

2.3.7 Homogeneity study

A CRM certified for its DNA sequence is certified for identity. The sequence identify defines the structure. The homogeneity of the material is therefore determined by the purity of the material.

However, additional homogeneity studies should verify the homogeneity in view of the intended use of the CRM.

a) This could e.g. concern the DNA amount, justifying to measure the mass of double stranded DNA in a representative number of vials by fluorescence. The obtained data can be evaluated like a homogeneity study (e.g. as outlined in ISO Guide 35[1]), proving that the mass of DNA in each vial is not significant different from each other (95 % confidence level).

b) For gDNA, the intactness of the DNA might be of relevance and a number of vials could be analysed by gel electrophoresis. A single band of DNA confirms a reasonable intactness of the gDNA in each vial.

c) PCR amplification in different vials should be checked, when the CRM is intended for PCR. For real-time PCR, this could include the monitoring of the crossing point threshold (Cp) and/or the melting temperature. This would contribute to the confirmation of the sequence identity (within the DNA sequence defined by the primers).

Uncertainties related to homogeneity studies should not be above a level at which the random selection of a vial would have an impact on the performance. A CRM certified for its DNA sequence and intended to be used as positive control in PCR applications needs, e.g. to contain in each unit enough of the DNA so that the PCR result is not negative. Impurities may influence the suitability of the material if they interfere with the PCR but are not relevant if they e.g. concern not targeted DNA and/or background DNA added to enhance the stability of the targeted DNA in the CRM.

2.3.8 Minimum sample intake

A CRM certified for its DNA sequence is often a pure material. Therefore, classical studies designed to investigate the minimum sample intake[12] are not applicable.

Instead the suitability and homogeneity studies, in which the CRM was used for its intended purpose, can be used to demonstrate that a certain amount of the CRM is sufficient to obtain the expected results. A minimum sample intake for a given purpose can be recommended. The establishment of a minimum sample intake in this case often resembles the typical sample intake area, rather than the minimum (possible) sample intake.

2.3.9 Short-term and long-term stability

A CRM can be certified for its DNA sequence. The sequence identify is unlikely to change, even DNA degradation would strictly speaking not impact the identity.

However, during short-term stability studies the transport stability and during long-term stability studies the to-be expected storage stability in view of the intended use of the CRM should be investigated. Based

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on the outcome, transport conditions and shelf-lives for specified storage conditions, not influencing the intended use should be selected.

Uncertainties related to stability studies should ensure that the material can still be used without impact on the performance after a certain time and/or exposure to specified temperatures.

2.3.10 Identification probability

No uncertainty concept exits for identity. The probability to misidentify for instance the DNA sequence of a material is often very low. To demonstrate this, the probability should be calculated that two sequencing procedures, independently performed in different laboratories, generated identical results by forward and backward sequencing.

However, one has to be aware that the DNA sequence information is used to 'identify' the material. This identification can be based on established classification schemes, which in itself can be subject to changes. It therefore should be mentioned which classification scheme has been applied at which time.

The probability for wrong identification in some specific areas can be rather high. e.g. if the DNA sequences are very similar, if crossings occur and if the availability of DNA sequence information is very limited. However, it should be carefully discriminated between the probability of a wrong outcome of DNA sequencing and the probability of a wrong identification because of a classification scheme which was set up on limited DNA information.

2.3.11 Documentary and metrological traceability

The certified identity is often based on a documentary traceability to a document linking the DNA sequence to a certain identity. Additionally, a metrological traceability exists for the confirmation step (such as dye terminator cycle sequencing or PCR measurements) carried out on the DNA sequence.

Beside the metrological traceability, the traceability (trackability) to a classification scheme is of interest.

3 Organic reference materials for qualitative analysis

3.1 General

Chemical measurement, whether it is quantitative or qualitative, requires reference materials for which the analyte of interest has been correctly identified. Laboratories are encouraged to purchase appropriately certified reference materials from an accredited reference material producer (RMP), providing confidence that the identity of their product has been correctly assigned. Due to the lack of reference materials certified for identity, laboratories are often forced to purchase chemicals from reputable chemical suppliers or simply use a sample "found on the shelf" and put their faith in the manufacturer's labelling and/or associated paperwork. Regardless of the source of the material, it would be considered prudent to perform in-house cross checks before embarking upon a testing program with a potentially incorrectly labelled reference material. Most modern day analytical laboratories will have access to mass selective detectors, linked to either liquid chromatography (LC-MS) or gas chromatography (GC-MS) equipment, which provide a fingerprint of the compound in hand. Specialized laboratories may also have access to infrared (IR) spectrometers, which can provide further structural information. Demonstrated equivalence with literature spectra is generally considered sufficient for structural confirmation. In cases where literature data are limited or non-existent, the analyst would need to exercise a lot more care when interpreting the structural information. The analyst also needs to appreciate the limitations of these techniques, particularly when considerations such as regio- and stereochemistry are important. In such cases, nuclear magnetic resonance (NMR) spectroscopy is recommended, as this technique provides a definitive structural analysis. On the downside, this introduces the need to access an NMR spectrometer and suitably qualified staff to interpret the spectra, which may be difficult for many laboratories. Fortunately, many university chemistry departments now offer this service.

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