



GUIDANCE DOCUMENT ON THE USE OF REFERENCE MATERIALS IN GENETIC TESTING

Project : NoE EuroGentest, Unit 1, WP1.6

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Summary

The aim of this guidance document is to provide a short and user-friendly support to genetic testing laboratories. It is based on internationally harmonised basic concepts and terminology for quality assurance and control (QA/QC). Relevant terms of the International Organization for Standardization (ISO) and the International Vocabulary of Metrology (VIM) are explained and references to other expert texts are provided. The document includes discussion and clarification of several often misunderstood issues. It takes into account the current scientific knowledge and the existing technological capabilities. This document may be revised, when appropriate, to take into account progress in science and technology and constitutes a deliverable of the NoE EuroGentest.

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Glossary

CE	Marking according to the EU IVD Directive)
CDC	Centers for Disease Control and Prevention (USA)
CITAC	Co-operation on International Traceability in Analytical Chemistry
CRM	Certified Reference Material
CLSI	Clinical Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EC	European Commission
EEC	European Economic Community
EQA	External Quality Assurance
EU	European Union
FDA	Food and Drug Administration (USA)
GB	Great Britain
GUM	ISO Guide to the Expression of Uncertainty in Measurement, ISO, Geneva 1995
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IQC	Internal Quality Control
ILAC	International Laboratory Accreditation Cooperation
IRMM	Institute for Reference Materials and Measurements, Joint Research Centre, European Commission
ISO	International Organization for Standardization
IVD	In vitro Diagnostics
LOD	Limit of Detection
LOQ	Limit of Quantification
MLPA	Multiplex Ligation-dependent Probe Amplification
NIH	National Institutes of Health (USA)
NIST	National Institute of Standards and Technology (USA)
OECD	Organization for Economic Cooperation and Development
PAP	Pyrophosphorolysis-Activated Polymerization
PCR	Polymerase Chain Reaction
PHRED	PHRED is a base-calling program for automated sequencer traces
QA	Quality Assurance
QC	Quality Control
QCM	Quality Control Material
RM	Reference Material
REMCO	ISO Committee on Reference Materials
RFLP	Restriction Fragment Length Polymorphism
VIM	International Vocabulary of Metrology - Basic and General Concepts and Associated Terms
WHO	World Health Organization

1. Introduction

Genetic tests can be highly predictive for the future health of the individual and can be carried out at any stage of life even in the embryo before implantation. They are relevant to healthy people as well as those showing symptoms of an unhealthy condition and may also have important implications for the relatives of the person tested. Given the rapid translation from research into clinical practice, research laboratories play a valuable role in service provision worldwide. The genotype established by a single laboratory test is usually not repeated and forms a permanent part of the medical record of the patient. Consequently, molecular genetic testing requires a high level of data reliability based on proper quality assurance of testing laboratories as well as of their measurement and testing procedures. As the field develops, the need for appropriate reference materials which are required to establish this reliability becomes increasingly urgent. The European Union (EU), the United States Food and Drug Administration (FDA) and National Institutes of Health (NIH), the Organisation for Economic Co-operation and Development (OECD), the World Health Organization (WHO), the Clinical Laboratory Standards Institute (CLSI) and other international bodies have realized the complexity of the problem and the importance of having rapidly clear guidelines addressed to the concerned parties.

The EU regulates genetic tests through the *in vitro* diagnostic (IVD) medical devices Directive (98/79/EC) which covers commercial diagnostic tests, and the 92/42/EEC Directive on medical devices. The IVD Directive deals with all aspects of safety and performance, taking on board the need for common technical specifications such as sensitivity. Its main purpose is to introduce harmonised controls on these IVDs throughout the EU.

2. Metrological Context and Terminology

The metrological terms and specific vocabulary used in this document are summarized in Annex 1.

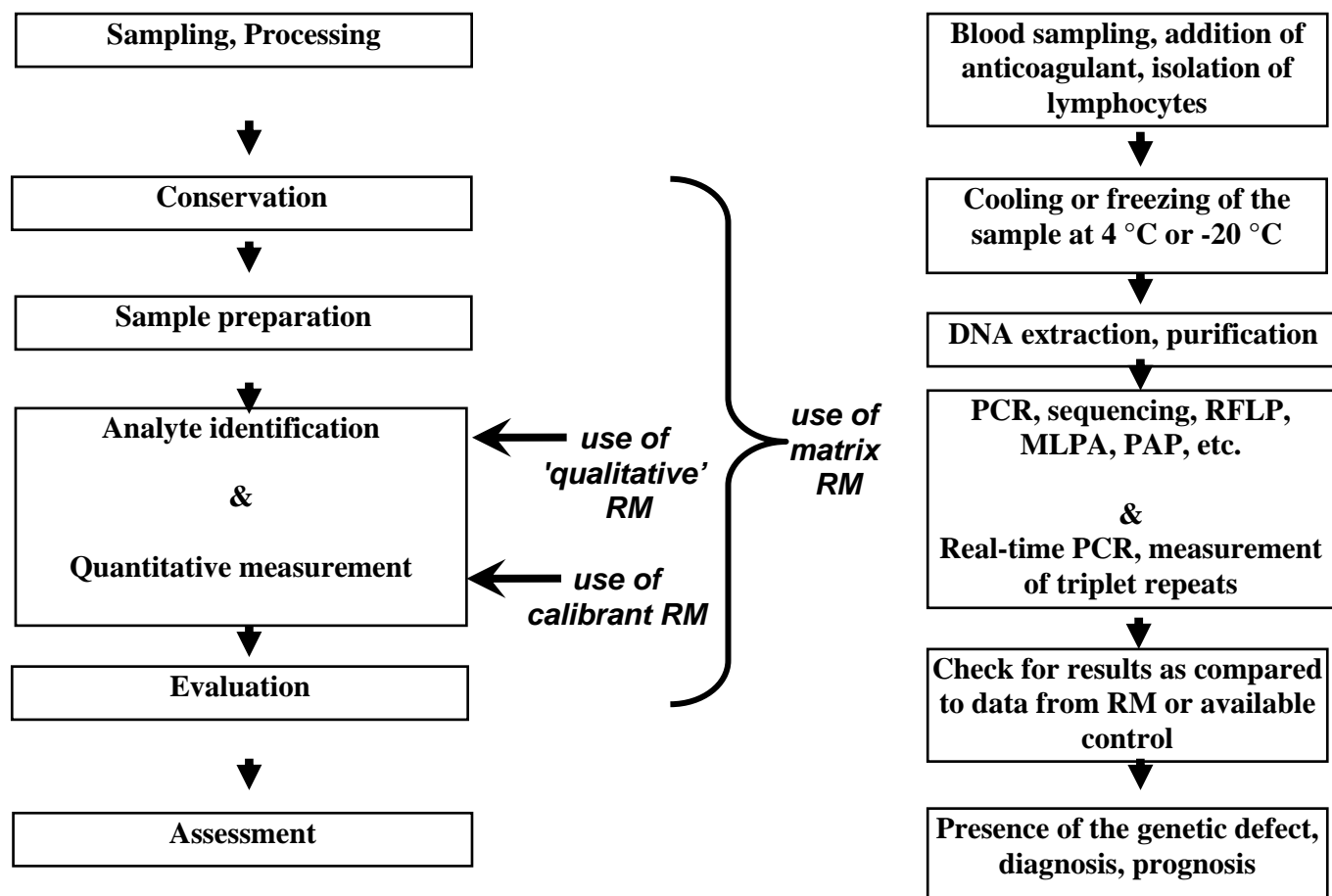
The type of reference material which is required to perform a proper analysis of the sample of interest depends on the analytical problem. An analysis which is directed to qualitative properties of a sample (such as the chemical/biological identity of a sample component) needs a RM which allows comparison of this qualitative property in the sample and in the RM during the application of the same measurement or testing procedure. A typical example would be a RM consisting of plasmid DNA with a well defined base pair sequence used for the quality assurance of the DNA sequencing procedure of an unknown genomic DNA fragment. As the synthetic DNA RMs lack the complexity of the human genomic DNA and are sometimes designed to work with a dedicated platform, they might not perform identically to patient DNA. Therefore these materials have to be tested in ring trials, proficiency studies, or EQA schemes so that their fitness for purpose is demonstrated.

On the other hand, most of the steps in quantitative measurements have to be calibrated because of the lack of completely known mathematical equations to calculate the relation between the targeted quantity in the sample and the measurement signal. Such calibration materials are almost indispensable for chemical and biochemical measurements. Moreover, reliable measurement results on the majority of the real-world samples can only be obtained if appropriate quality control measures are applied for most steps of the analytical process (Figure I). Therefore, so-called matrix RMs are developed which mimic as closely as possible the real sample and allow not only the control of the quantification step, but also of other steps such as sample preparation.

The typical applications for reference materials are:

- method development and validation, in particular evaluation of trueness and evaluation of measurement uncertainty
- calibration
- proof of method performance, such as statistical quality control (via control charts etc.), establishing traceable results and qualification of equipment
- proficiency testing, i.e. training and verification of the competence of laboratories

Figure I: Steps involved in the analytical process in general (left) and corresponding steps in genetic testing (right)



Unfortunately a multitude of names is presently used for designation of RMs. Depending on the field of analytical activity, on the awareness of international guidelines, concepts of quality assurance and metrology and even on regional peculiarities one can find different terms for such RMs. Examples are: measurement standard, laboratory standard, reference standard, analytical standard, reference substance, standard material, quality control material, proficiency testing material, laboratory control material, laboratory reference material or calibration material. Part of the terminology confusion seems not only to originate from different traditions of the various analytical/measurement communities, but also from the different understanding of underlying concepts. For instance, the interrelation between the intended use for a RM in a given measurement procedure and the required minimum material characteristics together with the distributed RM information is often neglected.

Recently the ISO Committee on Reference Materials (ISO REMCO) approved new definitions for "RM" and "CRM" (1). The term 'reference material' is now defined as follows: "Material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process.

NOTE 1: RM is a generic term.

NOTE 2: Properties can be quantitative or qualitative, e.g., identity of substances or species.

NOTE 3: Uses may include the calibration of a measurement system, assessment of a measurement procedure, assigning values to other materials, and quality control.

NOTE 4: An RM can only be used for a single purpose in a given measurement".

The first note mentions now explicitly that the expression ‘reference material’ is an “umbrella term” for various materials which are needed in measurement procedures in addition to the sample to be analyzed. Consequently one could consider the different RM types as members of a family (2).

Obviously, all materials possessing the characteristics of adequate homogeneity and stability required for quality control of a given measurement belong to this RM family. The ones which are not accompanied by a certificate are often simply called non-certified reference materials. But many other terms such as in-house materials, laboratory control materials or laboratory reference materials are also used. Here the term “Quality Control Material (QCM)” is favored for this subgroup of RMs for which only the material characteristics of homogeneity and stability fit for the intended use are proven. QCMs may support one or more applications from the wide range of both internal and external quality control measures. But they are not sufficiently characterized to be used for method calibration, trueness control or to provide metrological traceability of a measurement result.

Another subgroup of RMs is formed by the certified reference materials (CRMs). They are now defined as “reference material, characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability. NOTE 1: The concept of value includes qualitative attributes such as identity or sequence. Uncertainties for such attributes may be expressed as probabilities.

NOTE 2: Metrologically valid procedures for the production and certification of reference materials are given in, among others, ISO Guides 34 and 35.

NOTE 3: ISO Guide 31 gives guidance on the contents of certificates.”

That means a reference material belongs to this subgroup if in addition to the QCM characteristics a certificate is provided, giving a certified value with its uncertainty and a stated metrological traceability. Further details about these minimum quality characteristics of CRMs are explained in the corresponding ISO standards (3, 4, 5, 6, 7).

The other RM subgroup is composed of the materials used for calibration. They are often denoted as analytical standards, reference standards or simply calibration materials, but usually not explicitly recognized as “reference materials”. Such products, in particular calibrants consisting of pure chemical substances or solutions thereof, are described by some scientists or organisations as having a “higher metrological order” than CRMs. This misperception originates from a mixing of classification systems. As regards the material characteristics necessary and the information provided, materials for calibration have to be sufficiently homogeneous and stable so as to ensure that the assigned property value and its uncertainty are valid for any calibration sample used according to the given specifications. Therefore, they fall under the RM definition given above and the term “calibrant” is used here for such materials. The necessary additional features of a calibrant in comparison to a QCM are a stated property value with an uncertainty and a metrological traceability of the property value. These characteristics are not always completely fulfilled by various materials nowadays used for calibration in different measurement communities or laboratories but that means only that insufficiently characterized materials are used for this purpose and it does not invalidate the principally required minimum quality characteristics for calibrants, in particular with respect to known uncertainty and traceability of the value used for calibration.

For establishing harmonized references for measurement results of measurands for which their metrological traceability cannot be straightforwardly established to an independent measurement scale such as the SI, some measurement communities have started to set up so-called reference measurement systems. They are composed of reference methods, reference materials and reference laboratories. To define and harmonize a measurement target, the respective organisation or network, such as IFCC (International

Federation of Clinical Chemistry and Laboratory Medicine) in the field of clinical and laboratory medicine, agrees on a common reference method. The measurand therefore is completely defined via the method. The measurand is not simply derived from a well defined molecular entity only (such as concentration of glucose in blood), but is defined as the signal obtained when a special set of instructions is followed. This has to be accompanied by one or more reference materials which allow the installation of the corresponding reference method in the laboratory of interest and the performance of the method to be checked regularly. Most of these materials are certified reference materials, but there may be also sufficiently qualified reference materials which do not officially come with a full certificate as described in ISO Guide 31. Nevertheless these RMs could be qualified to be used at the highest metrological level which can be achieved for the time being. An illustrative representation of corresponding traceability chains for in-vitro diagnostics is published and briefly discussed in ISO 17511 (7). The reference measurement system is completed by the establishment of reference laboratories that maintain the method performance and the production of the most accurate results achievable at present.

Customers often do not fully use the potential of the purchased RM. One should realize that CRMs are not only a physical product in a bottle or ampoule, but that they are accompanied by important information on the material characteristics. CRMs which fulfil the requirements of ISO Guides 34 and 35 are delivered with a certificate which contains in addition to the certified value information about the uncertainty of the certified value and information about the metrological traceability. Moreover, this certificate includes instructions for use, storage conditions, expiration date of the certificate and either directly or via a link to other documents information about the procedures of its characterisation. The recommended content for CRM certificates is summarized in ISO Guide 31 (8). RM producers such as IRMM make an even more comprehensive description of the whole CRM preparation and certification process publicly available (www.irmm.jrc.be).

It is self-evident that the user of a CRM does not have to care about the details of the CRM characterisation, if she/he can be confident that scientifically sound and internationally accepted approaches have been followed by the RM producer. However, it would be advisable that the user checks if this was indeed the case and if proper statements on metrological traceability and uncertainties of the certified values, which include all necessary components, are provided as information together with the material. With the increasing implementation of quality management systems and accreditation of testing laboratories, the critical consideration of the confidence in the producer of the RM used by a laboratory gains importance as well. Comparable to developments for the laboratory service of measurements during the last 10 years, there are now demands for demonstrated competence of RM producers through benchmarking against internationally agreed and harmonized criteria rather than accepting 'designated' competence largely based on self-declaration or traditional recognition. An increasing number of RM producers is seeking for accreditation according to ISO Guide 34.

2.1. Fitness for purpose

The suitability of a RM is based on the fact that the rigour with which an assessment needs to be conducted depends on the criticality of the measurement, the level of the technical requirement and the expected influence of the particular RM on the validity of the measurement. A formal suitability assessment is required only if the choice of a RM can be expected to affect the measurement results significantly.

Factors to be considered include the following:

1. The suitability of a RM depends on the details of the analytical specification. Matrix effects and other factors such as the concentration interval can be more important than the uncertainty of the certified value (9).

The factors to consider include:

- Measurand
- Measurement interval (concentration)
- Matrix match and potential interferences
- Sample size
- Homogeneity and stability
- Measurement uncertainty

2. The validity of the 'certification' and uncertainty data, including conformance of key procedures with ISO Guide 35 and other ISO requirements (10, 11).

3. Track record of both the producer and the material. For example, when a RM has been subjected to an interlaboratory comparison, cross-checked by different methods, or successfully used by a number of laboratories.

4. Availability of a certificate and report conforming to ISO Guide 31.

5. Demonstrated conformance of the production of the RMs with international standards such as ISO Guide 34 or corresponding ILAC requirements (12), or compliance of the measurement of property values with ISO/IEC 17025 (13) requirements.

All or some of the requirements may be specified in the customer's analytical instruction sheet, but often it will be necessary for the analyst to use professional judgement. Finally, quality does not necessarily equate to small uncertainty and fitness for purpose criteria need to be used.

2.2. Metrological Traceability

Traceability of measurement results on a material is pivotal to the use of this material. Indeed, the planning of the measurements related to the characterization of the material depends on the standard to which traceability should be established and the means by which traceability will be established. To be able to establish traceability of a value to a certified value of a stated reference material, all measurement results that are used for the assignment of this value (and its uncertainty) need to be traceable to this stated reference (Figure II).

Three different kinds of traceability can be envisaged:

- traceability to the international system of unit (SI): this option is the best as the values are independent of any validated method and artefact. They are universally valid.

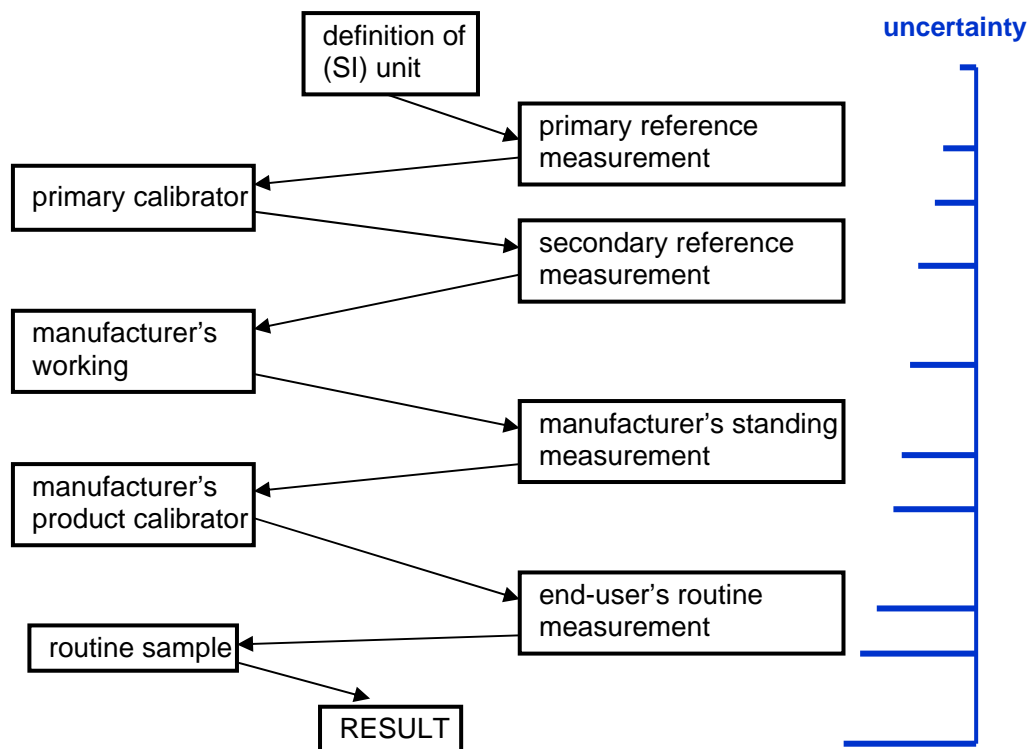
- traceability to a method: the values/properties assigned to the material are only valid when a specific measurement protocol is strictly followed. Of note, when establishing traceability to a method of the results of a homogeneity or stability study, all measurements must be performed strictly according to the predefined measurement protocol of this method. As the analyte is defined via the method, results from other methods are not related to the assigned value/property. If any method other than the specific one has to be used, the reasoning why the result of this other method should be traceable to the result of the specific method must be laid down in the (certification) report.

- traceability to an artefact (to a standard, to a particular instrument): this kind of traceability can be dependent or independent of a specific method. Of note, when establishing traceability to an artefact independent of a method, it must be proven that the methods used in the homogeneity or stability study are validated, and that all measurement results are traceable to this artefact. The requirements for method validation include the following points: selectivity and interferences, correctness, and working range. When establishing traceability

to an artefact dependent on a specific method, it must be proven that this specific method is used in the homogeneity or stability study, validated, and that all measurement results are traceable to this artefact. If any method other than the specific one has to be used, the reasoning why the result of this other method should be traceable to the result of the specific method must be laid down in the (certification) report.

By nature, the most common traceability statements to be achieved for genetic testing purpose are traceability to a method and to an artefact.

Figure II: Schematic representation of a traceability chain



In clinical chemistry, the term "value transfer" is used to denominate calibration of a property value in one material against the same property in another material. Different calibration or value transfer procedures are possible and depend on the availability of a suitable reference material and its commutability within a measurement procedure (14). The characteristics of an RM for use in a value transfer are:

- that characterization of the material is such that it satisfies the definition of the measurand
- that it possess a sufficiently large property value to allow the assay of several dilutions within the working range of the method
- that commutability/fitness for purpose has been proven
- that validated stability and homogeneity of the material are available

If these requirements are met, and the value transfer method is adequate, the target material can be traceable to the RM.

2.3. Estimation of bias

Estimation of bias (the difference between the measured value and the true value) is one key element of method validation. Appropriate RMs can provide valuable information on that, within the limits of the uncertainty of the certified value(s) and the uncertainty of the method being validated. Clearly the RMs must be within the scope of the method in terms of matrix type, analyte concentration etc. and ideally a number of RMs covering the full range of the

method should be tested. Where minor modifications to a well-established method are being evaluated then less rigorous bias studies (fewer replicates, larger range of concentrations) can be used. In addition, replicate measurements of the RM, covering the full range of variables allowed by the method being validated can be used to estimate the uncertainty associated with any bias, which should normally be corrected for (15).

3. State of the Art

3.1. In Vitro Diagnostic Medical Devices Directive (98/79/EC)

The main purpose of the In Vitro Diagnostic Medical Devices Directive (98/79/EC), the Medical Devices Directive 93/42/EEC and the Active Implantable Medical Devices Directive 90/385/EEC, now implemented in the Member States, are to create a single market. This is done by introducing harmonised and statute-based controls to regulate the safety and performance of medical devices throughout the EU.

A **medical device** is defined as "any instrument, apparatus, appliance; material or other article, whether used alone or in combination, including the software necessary for its proper application; intended by the manufacturer to be used on human beings for the purpose of:

- diagnosis, prevention, monitoring, treatment or alleviation of disease,
- diagnosis, monitoring, treatment; alleviation of or compensation for an injury or handicap,
- investigation, replacement or modification of the anatomy or of a physiological process,
- control of conception;

and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means".

The term ***in vitro* diagnostic medical device** refers to "any medical device which is a reagent, reagent product, calibrator, control material, kit, instrument, apparatus, equipment, or system, whether used alone or in combination, intended by the manufacturer to be used *in vitro* for the examination of specimens, including blood and tissue donations, derived from the human body, solely or principally for the purpose of providing information:

- concerning a physiological or pathological state, or
- concerning a congenital abnormality, or
- to determine the safety and compatibility with potential recipients, or
- to monitor therapeutic measures".

The CE mark grants that the device satisfies the relevant essential requirements, that it is fit for its intended purpose as specified by the manufacturer, and that the product can be freely marketed anywhere in the EU without further control.

3.2. Patent issues related to reference materials (RMs) for genetic testing

The European Directive 98/44/EC allows gene patents under certain circumstances as specified in Article 9: "The protection conferred by a patent on a product containing or consisting of genetic information shall extend to all material in which the product is incorporated and in which the genetic material is contained and performs its function." The patent law specifies the acts that are prohibited to third parties (which could mean those developing, producing or using RMs). Generally, the patent owner has the right to prevent any third party from making, using, offering for sale, selling or importing the patented product. The actual meaning of patent claims is ultimately decided by the courts. There are no general patent rules for the production of a reference material for genetic testing and it is common to proceed on a case by case basis.

The use of short sequences from a gene patented for its expression of a novel protein will not infringe the patent, as in this particular case the function of the novel protein is patented and not the shorter DNA fragments of that gene. But patents on gene sequences can create a barrier to producing RMs. It is always advisable to verify the territoriality of the patent and its expiration date, or to get a licence from the patent owner.

One way to prevent the patenting of newly-discovered genes, newly-developed genetic tests or newly-characterized reference materials that can be important for public health is to put the information into the public domain, e.g. by publication in a scientific journal.

3.3. The EuroGentest project

EuroGentest is an international Network of Excellence funded by the European Commission which is looking at various aspects of genetic testing - quality management, information databases, public health, ethics and legal issues, new technologies and education. EuroGentest aims at test development, harmonization, validation and standardization for genetic testing in Europe. As technologies used for molecular genetic testing begin to enter the mainstream of clinical practice, the need for appropriate reference materials that could guarantee the development, validation and harmonization of these methods becomes increasingly urgent. Accordingly, EuroGentest partners from the EU and the Centers for Disease Control and Prevention (CDC) of the USA gathered together in two International Symposia on Reference Materials for Genetic Testing in 2005 and 2007 to discuss key issues such as regulations, current RM availability, development and prioritisation of future needs. The present document contributes to the deliverables of the work package 1.6 of Unit 1 dealing with reference systems.

3.4. OECD position

The Organization for Economic Co-operation and Development (OECD) has proposed Guidelines for Quality Assurance in Molecular Genetic Testing (16). The Guidelines offer principles and best practices for human genetic testing by encouraging high quality laboratory practices in the collection and handling of samples and data, result reporting, education and training. They address genetic testing for variations in germ line DNA sequences or products arising directly from changes in heritable genomic sequences that predict effects on the health, or influence the health management, of an individual. They focus on molecular genetic testing for the diagnosis of a particular disease or condition and predictive genetic testing often carried out before any clinical signs of the disease or condition appear. They are relevant to tests for heritable DNA variants that predict the response profile of an individual to a drug or course of therapy and that affect susceptibility to disease, patient prognosis, counselling, treatment and family planning. Besides accreditation that can guarantee quality assurance, the guidelines encourage as well the validation of the tests to be performed, the monitoring of the quality of laboratory performance using proficiency testing schemes, the quality of test results reporting and the education and training for laboratory personnel.

In addition, parts of these guidelines are also relevant and applicable to aspects of clinical cytogenetics testing and biochemical genetic testing. However, they are not designed to address directly the areas of testing for somatic mutations, variants important in tissue matching, genetic analysis of pathogenic organisms and identity testing, though all share related technologies.

In conclusion, the guidelines are meant to encourage quality assurance systems for human genetic and genomic testing, guarantee the international exchange of clinical samples and

access to data on rare disease testing. The ethical and legal principles set out in international declarations and agreements and the diversity of national jurisdictions have been recognised during their development.

3.5. Availability of Reference Materials

The availability of certified reference materials is a prerequisite for a thorough method validation and supports the development of reliable commercial genetic testing kits. In general RMs are essential for internal quality control and the performance of External Quality Assurance Schemes (EQA), including the monitoring of test performance, the detection of errors in the testing procedure and the validation of any test intended for patient testing. Analysis of appropriate positive and negative samples for the genotype to be detected can establish or verify sensitivity, specificity, and other performance characteristics for the test.

The basic requirement for routine testing is to include well-characterized positive and negative samples in each run of patient specimens. Presently, most genetic tests are developed in-house by individual laboratories and therefore harmonization of the control samples and of the measurement procedures is needed.

The final goal of a genetic test is the potential identification of variants or abnormalities in a nucleic acid sequence such as mutation(s), translocation(s), duplication, amplification and deletion(s). With the exception of copy number variations, the quantification of the target sequence modification is not needed as the expected diagnostic result consists of a yes/no answer about the presence or absence of the modification. Therefore potential RMs have to provide (certified) properties which are more of "qualitative" than "quantitative" nature. High quality materials with a certified DNA sequence, for which homogeneity among the molecules, stability and commutability or suitability of the materials have been proven, are required. At present the availability of RMs for genetic testing is rather limited.

Examples of available CRMs for genetic testing are the three RMs produced by IRMM (EC) for the human Prothrombin/Factor II G20210A mutation detection (plasmids).

Other certified reference materials available from NIST (USA) (called standard reference materials by NIST) are the Fragile X human DNA triplet repeat standard RM 2399 and Human Y-chromosome DNA profiling standard RM 2395 (NIST).

NIBSC (GB) has also produced several materials (called international standards) for genetic testing on behalf of WHO, but the homogeneity of those has not been tested. Therefore, although the stability of these materials was tested via accelerated degradation schemes (up to 6 months storage at 45 °C and 56 °C), they cannot be called RMs according to the definition stated in ISO Guide 34 (3).

CDC (USA) is coordinates a program for the development and characterization of cell lines derived from patients' blood suffering from various genetic diseases and provides either the cell lines, either a dried cell pellet as RM.

Other private companies (MMQCI, Roche, etc.) have also developed controls in their kits for testing but the requirement of stability and homogeneity testing are not always straightforward.

The EuroGentest website (www.eurogentest.org) also lists RM producers and available products for genetic testing.

3.6. Conclusion

Genetic testing is a burning issue as the results of the tests can have consequences not only for the individuals tested but also for their relatives. Therefore legal and ethical considerations are applicable (regulated, among others, by the IVD Directive 98/79/EC in

Europe). In addition, appropriate quality assurance of the testing is a pre-requisite for achieving confidence in the results within and between laboratories. Quality management and harmonization of the tests are investigated in Europe by the NoE EuroGentest and the OECD has recently issued guidelines for quality assurance in molecular genetic testing (16). Although the field of genetic testing is evolving rapidly, the availability of RMs required for method development and validation is still limited.

4. Selection criteria for reference materials according to their use

General guidance is provided, among others, in ISO Guides 32 (17) and 33 (18) and Eurachem CITAC Guide (19). Therefore, only the main aspects and some specific recommendations are summarized hereafter and in Annex 2.

4.1. Method Development and Validation

The successful installation of a new method in a laboratory includes the following steps: method development, implementation, validation and performance monitoring. It is necessary to establish that the signal produced at the measurement stage or other measurement property, which has been attributed to the analyte, is only due to the analyte and not from the presence of a chemically or physically similar entity. This is confirmation of identity. Whether or not other components will interfere with the measurement of the analyte will depend on the effectiveness of the isolation stage and the specificity of the measurement stage. A method should be validated to verify that its performance parameters are adequate for use for a particular analytical problem. Some validation protocols confuse confirmation of identity with repeatability. Whereas evaluation of repeatability requires the measurement to be performed several times by one technique, confirmation of identity requires the measurement to be performed by several, preferably independent techniques.

Method validation gives an idea of a method's performance capabilities and limitations which may be experienced in routine use while the method is in control (that is the method is performing the way expected).

4.2. Diagnostic Tests Development and Validation

In the case of diagnostic tests for genetic testing, one can identify different phases:

- a design phase including a review of the literature, of the clinical utility of the test, a detailed description of the method, the required staff and the necessary equipment
- a production phase describing the product specifications, the environmental conditions that might influence its integrity
- an initial technical validation phase including the use of RMs and/or of reference measurement procedures (e.g. for verification of patient status), determination of the uncertainty (if applicable), method specificity and sensitivity
- a permanent quality monitoring phase that reviews annually the performances and properties of the product, for instance using an EQA scheme and/or regularly reference materials.

4.3. Calibration (for quantitative methods)

Normally a pure substance RM is used for calibration of the measurement stage of a method. Other components of the test method, such as sample preparation, are not covered. Loss of analyte, contamination and interferences and their associated uncertainties must be addressed as part of the validation of the method, for example using an internal standard. The uncertainty associated with the RM purity will contribute to the total uncertainty of the measurement. In the field of genetic testing, purified plasmidic or genomic DNA can be considered as pure substance calibrant.

The use of matrix RMs for calibration of the complete analytical process constitutes a scenario close to real samples, but the most important aspect is that the analyte in RMs must be in a form which behaves similar in the measurement procedure to the real samples (20). However, RMs from the same source should not be used for both calibration and the ongoing

assessment of a measurement procedure. Using the same RM for both applications would create a vicious circle and would not allow the identification of calibration errors. For this reason, the ISO Guide 35 states that an RM can only be used for a single purpose in a given measurement.

The ISO Guide 32 and reference (11) provide additional useful information.

4.4. Quality Assurance and Quality Control (QA & QC)

Materials for statistical quality control are used for a longer period of time to allow conclusions about the performance and potential changes of a method or the laboratory personnel to be drawn from control charts. RMs used in this case should be characterized with respect to homogeneity, stability, and their property value(s). Similar requirements apply to samples used to establish how measurements made in different laboratories agree.

In the case of proficiency testing, homogeneity of the distributed subsamples is essential and sample stability within the time-scale of the exercise must be assessed and controlled.

4.5. Interlaboratory studies

Each interlaboratory study includes the distribution of various units of samples to a group of participants. Such studies are organized for proficiency testing of laboratories, method validation or method standardization or characterization of candidate RMs. The homogeneity of the materials used for such exercises is crucial as variations of results should only reflect the reproducibility of the method(s) and not the suitability of the material as reference.

4.6. Identity checks

In the case of the identification of a nucleic acid sequence, DNA sequencing can be considered as one of the more robust methods. For validation purposes, attention should be paid that the sequence of interest has been obtained using forward and backward sequencing primers spanning the same target region. The uncertainty can then be expressed as the probability of misreadings in the sequence of nucleic acids in the target, for example using a PHRED score.

4.7. In-house RMs

If commercial RMs are not available for the specific user need, one has to consider to prepare an RM internally. There are guides available (20, 21) to help the non-specialist laboratory to prepare their own RMs. Some of the key issues that need to be considered are: selection of materials (appropriateness, native material versus spikes, material preparation etc.), homogeneity testing, preparation and packaging (homogeneity, contamination, stability etc.), stability testing, characterisation studies, uncertainty estimation, documentation and QA, storage and further stability monitoring.

5. Application Guidance

Some of the frequently asked questions concerning DNA-based RMs and their answers are listed in Annex 3.

5.1. Quantitative measurements

As mentioned previously, in the field of genetic testing, most applications are qualitative. However, the assessment of copy numbers of nucleic acid sequences (revealing the presence of a gene amplification or deletion, of contaminating micro-organisms, of a residual disease (as in cancer monitoring) or the number of trinucleotide repeats in a gene (such as in the Fragile X disease) can be considered as quantitative applications.

The determination of the copy number, length or size of a nucleic acid sequence in a gene should be performed according to the guidelines given above and the results must include a stated uncertainty determined by statistics and the associated traceability chain. Some of the methods involved are real-time PCR, fluorescence in situ hybridization and sequencing.

Every property value assigned to a reference material must be accompanied by a statement on its metrological traceability. The uncertainty linked to an assigned value consists of contributions from homogeneity, stability and characterization studies. Each contribution is estimated individually and the squared contributions should be combined. An example of quantitative assessment of nucleic acid sequence copy number can be found in the literature (22) where the use of genomic and plasmid DNA were compared for the assessment of the DNA copy number ratio in matrix-based genetically modified organisms-derived CRMs.

The measurement uncertainty is linked to the individual measurement performed but not to a defined method as such. The uncertainty arises from both sampling and analysis, unless it could be that the sampling carried out is representative and that the sampling uncertainty can therefore be neglected. Two approaches can be followed in order to determine the contribution to measurement uncertainty: either considering all the individual uncertainties resulting from the individual steps involved in the measurement (i.e. DNA extraction, PCR reaction, calibration, analysis of results), either take into account the data obtained from the method validation and quality assurance (i.e. repeatability, reproducibility, interlaboratory comparison). A detailed description of the second approach for measurement using real-time PCR measurements is provided in (23).

Moreover, for quantitative results, the following steps should be followed in order to compare one's own measurement result with the certified value of a CRM.

The underlying principle is that one has to check whether the difference between the measured result and the certified value is larger than the expanded combined uncertainty of measurement and certified value.

This is done as follows:

1. Calculate the standard uncertainty of the certified value (u_{CRM}).

This is done by dividing the expanded uncertainty given on the certificate by the expansion factor (also stated on the certificate).

2. Estimate the measurement uncertainty (u_m) of the result. As a very rough approximation, the reproducibility standard deviation can be used.

3. Combine the two uncertainties using the following formula:

$$u_c = \sqrt{u_m^2 + u_{CRM}^2}$$

4. Check whether $2 \cdot u_c$ is larger than the difference between the certified and the measurement value. If this is the case, the measurement result agrees within the limits of the respective uncertainties with the certified values.

See http://www.irmm.jrc.be/html/reference_materials_catalogue/user_support/use.htm]

5.2. Qualitative measurements

Guidelines from EURACHEM/CITAC are under development for these types of measurements (24). For qualitative measurements in genetic testing, the uncertainty can be expressed as a probability of obtaining a mistake/misreading of a base in the sequence. The calculation of this probability will depend on the measurement procedure and should be as low as possible.

In most cases, genetic testing consists in the assessment of the presence or absence of specific nucleotide sequence(s) using analytical methods that focuses on sequence identity or property rather than quantity.

As an example, if bi-directional sequencing has been used for identification of a 500 bp DNA fragment, the probability of misreadings in the sequence as a first estimate could be calculated as $1/500 * 1/500 = 4 * 10^{-6}$. This probability could be reduced further by sequencing the sample molecules backwards and forwards several times and on different platforms. Therefore, an internal control with a well characterized or better with a certified sequence (such as a commercially available plasmid vectors) should always be run in parallel to the sequencing reaction of the samples. After analysis, the results should then be compared to the corresponding sequences available in databases in order to identify the variants and to estimate the trueness of the measurement result.

Although sequencing is a time-consuming and expensive method as compared to other genotyping procedures, it is still quite robust (25-27), and remains the reference method in case of dubious results. The other methods used for genotyping (RFLP, Southern Blotting, PCR-derived methods for allelic discrimination, real-time PCR, MLPA, PAP, etc.) focus on shorter sequences and allow only the characterisation of the small fragments amplified.

For DNA sequencing to become widely accepted as a reference method for SNPs, uniform DNA samples would have to be dispatched to several labs for sequencing, and the results should be similar using different platforms and chemistries on different days to fulfil internationally accepted requirements for the validation of reference methods. However, for the experimental determination of the probability of sequencing failures under the requirement of matching forward and backward sequencing, a very large number of sequencing experiments would have to be carried out to detect the expected low number of matching misreadings.

Considering that other genetic variations could be difficult to identify by sequencing, there would not be only one reference method for genetic testing but several, depending on the defect to be identified. The most important characteristic is that the method would be robust and not affected by small changes such as temperature, pH, etc, and validated using RMs for which the target DNA sequence concentration is in the range of what can be expected in the case of real samples. In addition, this method should have been tested using interlaboratory comparison studies.

5.3. Highlights of important aspects for good QC in a genetic testing laboratory

Recommendations can be found in the standards ISO 15189 and 17025 which are the basis for accreditation of laboratories. The following recommendations can only be used for the period in which the laboratories are preparing for accreditation. The referral to the OECD principles of Good Laboratory Practice (28) that establish the minimum quality assurance requirements necessary to ensure the validity of experimental results can also be useful in this respect.

The following is recommended:

1. A laboratory responsible/manager/director should be designated in order to co-ordinate the activities of the laboratory and personnel.

2. Installing a new instrument/new test method in the laboratory:

Upon arrival of a new instrument, an instrument file in which all related documents such as maintenance, problems, authorized users, performance qualification, etc. should be prepared as well as a work instruction for its operation. This standard operating procedure or work instruction will describe its aim and scope, as well as the definitions, the description, and the necessary steps related to maintenance, safety and responsibilities for the instrument. In particular, an instrument qualification (performed to make sure that the instrument was properly installed), and a calibration/operation qualification (to check whether the instrument is fit for its intended purpose and works according to specifications) will have to be performed and documented. The instrument qualification needs only to be repeated if the instrument has been moved or if there is some other significant change in the instrument's environment. The operation qualification should be performed regularly, as specified in the work instruction. A logbook should be associated to the instrument so that all performed analyses are recorded, as well as potential problems, calibrations, maintenance, etc.

Forms for sample analyses and associated to the work instruction can be prepared so that all parameters of the related experiment are registered.

If new methods are introduced in the laboratory, they should be validated, this includes determination of method performance parameters such as LOD, LOQ, linearity/working range (if applicable), repeatability, robustness, specificity, sensitivity, trueness (see also section 3.1 for validation of diagnostic tests).

3. Introducing new laboratory personnel:

Training on the instrument will have to be organized for the concerned staff.

The personnel that should operate the instrument will have to demonstrate their ability by measuring a RM with the required specificity and sensitivity, and, in case of quantitative measurements, for establishing analytical performance parameters such as LOD, LOQ, trueness,...Therefore, blinded CRM samples should be distributed and the supervising staff should compare the obtained results with the certified values (and their uncertainties).

4. Routine operation of the lab:

Once the method(s) has been validated and the instrument is operational, a control chart can be established by using QC samples (for which homogeneity and stability have been proven) available in sufficient quantities for repetitive analysis. The random variation in performance of the analytical method can be monitored by monitoring the analysed value of the QC sample, usually by plotting it in a control chart. Limits are set for the values on the chart (conventionally 'warning limits' are set at $\pm 2\sigma$ around the mean value, and 'action limits' are set at $\pm 3\sigma$ around the mean value). Each run should include an internal positive and negative control sample in order to assess the regular QC of the method. In addition, it would be advisable to measure regularly (as every 100th sample for instance or after a defined time period) a RM sample in order to compare the obtained results with the certified (assigned) RM value and introduce the measurement result in the control chart.

Major deviation of measurement results from the expected values/properties should be carefully scrutinized, investigated, recorded and reported to the laboratory responsible/director/manager.

5. The laboratory should participate regularly (when the possibility is offered by an EQA organizer) in an interlaboratory/proficiency testing exercise and demand from the provider a thorough evaluation of their performance afterwards.

6. The analysis requests, the reporting of the results and the retention of the records and the materials for a certain period should be guaranteed.

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ANNEX 1: Selected definitions relevant to genetic testing

Accuracy

The closeness of agreement between a test result and the accepted reference value.

Analyte

Bias

Difference between the mean measured value from a large series of test results and an accepted reference value (a certified or nominal value). The measure of trueness is normally expressed in term of bias

Calibrant/Calibrator

The necessary additional features of a calibrant in comparison to the general RM characteristics are identical to the ones of the certified reference material, except that the production of a certificate is optional. Calibrants should come with property values with sufficiently small measurement uncertainties and are used for direct calibration or other value transfer operations.

Calibration

Set of operations that establish, under specified conditions, the relationship between the values of quantities indicated by a measurement instrument or measuring system or values represented by a material measure or a reference material, and the corresponding values realised by standards.

Certificates and Supporting Reports

Ideally, a certificate complying with ISO Guide 31 and a report covering the characterisation and certification procedures, complying with ISO Guide 35, should be available for the certified reference materials. However, many RMs may not fully comply with ISO Guides 31 and 35 but equivalent information is available, providing credible evidence of compliance and can be considered as acceptable. Examples include the following: technical reports, trade specifications, papers in journals or reports of scientific meetings and correspondence with suppliers.

Certified Reference Material_(CRM, ISO Guide 35, 2006)

An RM characterized by a metrologically valid procedure for one or more specified properties, accompanied by a certificate that states the value of the specified property, its associated uncertainty, and a statement of metrological traceability.

Notes: 1) The concept of values includes qualitative attributes such as identity or sequence. Uncertainties for such attributes may be expressed as probabilities.

2) Metrologically valid procedures for the production and certification of reference materials are given in among others ISO Guides 34 and 35.

3) ISO Guide 31 gives guidance on the contents of certificates.

Commutability

1. Closeness of agreement between the mathematical relationship of the measurement results obtained by two measurement procedures for a stated quantity in a given material, and the mathematical relationship obtained for the quantity in routine samples (ISO 17511, 2003).

2. Property of a given RM demonstrated by the closeness of agreement between the relation among the measurement results, for a stated quantity in this material, obtained according to

two given measurement procedures, and the relation obtained among the measurement results for other specified materials (VIM 2007).

Note: The material in question is usually a calibrator, and at least one of the two given measurement procedures is usually a high-level measurement procedure. Therefore, one important application of a commutable reference material is assigning values to other materials.

3. Working definition: the equivalence of mathematical relationships between the results of different measurement procedures for a reference (or control) material and for native clinical samples.

Genetic testing

The UNESCO definition from the International Declaration on Human Genetic Data was used in this document, as the Network of Excellence EuroGentest is currently working on a definition. "A procedure to detect the presence or absence of, or change in, a particular gene or chromosome, including an indirect test for a gene product or other specific metabolite that is primarily indicative of a specific genetic change".

Limit of Detection (LOD)

Limit of detection is the lowest concentration or content of the analytes that can be detected reliably, but not necessarily quantified. LOD is generally expressed as the amount of analyte at which the analytical method detects the presence of the analyte at least 95% of the time (\leq 5% false negative results).

Limit of Quantification (LOQ)

The limit of quantification of an analytical procedure is the lowest amount or concentration of analyte in a sample, which can be quantitatively determined with an acceptable level of precision and accuracy.

Measurand (VIM, 2007)

The measurand is defined as the quantity intended to be measured.

In genetic testing, it could be interpreted as the presence of a particular DNA sequence in the patient's blood. However, due to the various steps required during the analysis of the DNA (starting from the DNA extraction procedure from blood to further analysis such as PCR, RFLP or sequencing), the target actually measured at the final stage of analysis might differ from the one intended to be measured at the beginning as byproducts or impurities might have been added/removed from the original blood sample during the analytical procedure.

Measurement Uncertainty

Parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand.

Notes:

1. The parameter may be, for example, a standard deviation (or a given multiple of it), or the half-width of an interval having a stated level of confidence.
2. Uncertainty of measurement comprises, in general, many components. Some of these components may be evaluated from the statistical distribution of results of a series of measurements and can be characterised by experimental standard deviations. The other components, which can also be characterised by standard deviations, are evaluated from assumed probability distributions based on experience or other information.
3. It is understood that the result of a measurement is the best estimate of the value of a measurand, and that all components of uncertainty, including those arising from systematic effects, such as components associated with corrections and reference standards, contribute to the dispersion.

Reference Material (RM, ISO Guide 35, 2006)

Material, sufficiently homogeneous and stable with respect to one or more specified properties, which has been established to be fit for its intended use in a measurement process.

Notes: 1) RM is a generic term.

2) Properties can be quantitative or qualitative, e.g. identity of substances or species.

3) Uses may include the calibration of a measurement system, assessment of a measurement procedure, assigning values to other materials, and quality control.

4) An RM can only be used for a single purpose in a given measurement.

5) A distinction between "pure substance" RMs and "matrix" RMs can be done. Matrix RMs for laboratory medicine are basically blood, urine, serum or other tissue samples which have been processed to reference material. The matrix is colloquially the surrounding substance around the analyte.

Sensitivity

The sensitivity of a method is a measure of the magnitude of the response caused by a certain amount of analyte.

The method should be sensitive enough in order to be able to detect/quantify with respect to the thresholds as provided in the relevant legislation.

Since sensitivity is method- and purpose-dependent it should be specified in the protocol. A reasonable goal for sensitivity is that required to meet levels specified in contracts, with a reasonable certainty that the level does not exceed the required limit.

Sensitivity as a term is used in two different ways - LOD and the slope of a curve. The use of the LOD is the preferred term to use as a measure of the ability of a method to detect a small amount of analyte.

Specificity

Property of a method to respond exclusively to the characteristic or analyte of interest.

Metrological Traceability (VIM, 2007)

Property of a measurement result whereby the result can be related to a stated metrological reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty

NOTES

1 For this definition, a 'stated metrological reference' can be a definition of a measurement unit through its practical realization, or a measurement procedure including the measurement unit for a non-ordinal quantity, or a measurement standard.

2 Metrological traceability requires an established calibration hierarchy.

3 Specification of the reference must include the time at which this reference was used in establishing the calibration hierarchy, along with any other relevant metrological information about the reference, such as when the first calibration in the calibration hierarchy was performed.

4 For measurements with more than one input quantity to the measurement model, each of the input quantities should itself be metrologically traceable and the calibration hierarchy involved may form a branched structure or a network. The effort involved in establishing metrological traceability for each input quantity value should be commensurate with its relative contribution to the measurement result.

5 Metrological traceability of a measurement result does not ensure that the measurement uncertainty is adequate for a given purpose or that there is an absence of mistakes.

6 A comparison between two measurement standards may be viewed as a calibration if the comparison is used to check and, if necessary, correct the quantity value and measurement uncertainty attributed to one of the measurement standards.

7 The ILAC considers the elements for confirming metrological traceability to be an unbroken metrological traceability chain to an international measurement standard or a national measurement standard, a documented measurement uncertainty, a documented measurement procedure, accredited technical competence, metrological traceability to the SI, and calibration intervals (see ILAC P-10:2002).

8 The abbreviated term 'traceability' is sometimes used to mean 'metrological traceability' as well as for other concepts, such as 'sample traceability' or 'document traceability' or 'instrument traceability' or 'material traceability', where the history ('trace') of an item is meant. Therefore, the full term of 'metrological traceability' is preferred if there is risk of confusion.

Trueness

The closeness of agreement between the average value obtained from a large series of test results and an accepted reference value.

ANNEX 2: Minimum Quality requirements for various types of reference materials

Material characteristics	Intended Use			
	Method development and validation	Interlaboratory comparison	Statistical quality control	Real sample to be analyzed
Matrix/concentration matching with real samples	As close as possible to real sample	Analyte/matrix combination as available in existing PT schemes	Matrix type should be comparable	
Minimum sample intake	Tested and found smaller than necessary for the tested method	Tested and found smaller than all of the methods potentially used	Tested and found smaller than the sample intake of the method	Sufficiently small for the method used
Between-unit homogeneity		Tested and found to contribute negligibly to uncertainty	Tested and found to contribute negligibly to uncertainty	Not applicable
Stability during transport		Tested and potential degradation during transport found negligible		Stable from sampling to the laboratory
Stability during storage	Tested and found stable during the duration of the study	Tested and found stable during the duration of the study	Long-term stability necessary	Stable for the duration of the analysis
Assignment of traceable values with uncertainty	Allows assessment of accuracy for method validation	Reference for traceability must be useful, uncertainty must be smaller than measurement uncertainty	At least a value traceable to the method must be assigned	
Certification	Guarantees above characteristics	Allow repetition of the study and constitutes reference for evaluation	Allows not only evaluation of trends but also an assessment of accuracy with every use	

ANNEX 3: Questions specifically related to DNA-based CRMs for genetic testing

Where do I get instructions on how to perform a PCR?

The description of specific detection methods is available in the literature. Detection methods used during the certification of a certified reference material are listed in the certification report. An example of certification of a reference material for genetic testing for the G20210A mutation in the prothrombin/FactorII can be found at the following address and typing as reference material code 490 (wildtype sequence), 491 (G20210A mutant) or 492 (heterozygous G20210A) (<http://www.irmm.jrc.be/rmcatalogue/searchResultrmcatalogue.do>).

Which DNA extraction method should I use?

Reliable results can only be guaranteed if an extraction method validated in a collaborative trial is used. Be aware that some extraction methods might generate inhibitors that can impair the subsequent detection reaction(s).

Is the sequence information corresponding to the genetic disease publicly available?

The wild-type sequence of a gene is in general accessible in the public database such as GenBank (www.ncbi.nlm.nih.gov), whereas the primer and probe sequences that target the specific region to be checked might be confidential or published and publicly available (in PUBMED for instance at www.ncbi.nlm.nih.gov). The sequence information of a gene might be patented in some countries, especially if the function of the gene is known. However, in some cases, the information can be used for public health reasons.

Are there reference materials available for the genetic test I am planning to perform?

There are several reference materials producers such as IRMM, NIBSC, NIST, CDC, and private companies. Consult the EuroGentest website (www.eurogentest.org) to find useful links to reference materials available and their producers.

How do I choose a reference materials for the genetic testing according to its formulation/presentation (according to CRMGEN project, FP5-funded project)?

Type	Similar to usual samples	Versatile	Stable	Economical to produce	Storage Cost	Ethical issues
Cell Line	+++	+++	-	+	-	+
Genomic DNA	++	++	+	++	++	+
Recombinant DNA	+	++	++	+++	++	++
PCR Product	+	++	++	+++	++	++
Synthetic DNA	-	-	++	++	++	+++

Key: +++, excellent; ++, very favourable; +, moderate; -, less favourable