EXPERT REVIEWS

Molecular diagnostics: harmonization through reference materials, documentary standards and proficiency testing

Expert Rev. Mol. Diagn. 11(7), 741–755 (2011)

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There is a great need for harmonization in nucleic acid testing for infectious disease and clinical genetics. The proliferation of assay methods, the number of targets for molecular diagnostics and the absence of standard reference materials contribute to variability in test results among laboratories. This article provides a comprehensive overview of reference materials, related documentary standards and proficiency testing programs. The article explores the relationships among these resources and provides necessary information for people practicing in this area that is not taught in formal courses and frequently is obtained on an *ad hoc* basis. The aim of this article is to provide helpful tools for molecular diagnostic laboratories.

Keywords: DNA • documentary standards • external quality assessment • genetic testing • infectious disease diagnostics • molecular methods • NAT • nucleic acid testing • proficiency testing • reference material

The need for standardization in molecular genetics & infectious disease testing

Molecular methods are used in a variety of ways to detect, quantify and sequence nucleic acids throughout many areas of laboratory medicine. These tests are used to diagnose cancer, to provide prognostic assessments, to aid in treatment selection and to monitor the efficacy of treatment through detection of minimal residual disease for each patient. Molecular tests are also used to detect bacterial and viral infections, estimate viral loads and guide selection of antibiotic and antiviral therapies. Finally, molecular genetic tests are used to identify patients who are affected with or carry genes predisposing to heritable genetic disorders. A wide range of analytical methods are employed in these tests, including DNA sequence analysis, quantitative PCR, molecular amplification methods that detect point mutations, deletions and duplications, and cytogenetic arrays.

There are molecular genetic tests for more than 1900 heritable disorders and an increasing number of infectious diseases and cancers. Commercial test kits are available for a few of the more common assays, such as cystic fibrosis, some pharmacogenetic tests, microbial detection and quantification of pathogens including HIV, cytomegalovirus (CMV) and *Clostridium difficile*. Many laboratories also develop their own assays. Significant concerns of regulatory agencies, manufacturers and laboratory directors focus on capacities to standardize and harmonize test results across method types, laboratory settings, assay applications (intended use) and geographic areas. Comparable assay results from all laboratories performing a test for the same analyte or measurand are critical in interpreting the clinical research in order to establish the utility of the tests and in the utilization of the results to make medical decisions.

Although results from proficiency testing/external quality assessment (PT/EQA) programs as well as interlaboratory specimen exchange studies indicate that there is a high degree of analytical agreement among laboratories for most tests, PT/EQA results for a few tests continue to show significant variability in quantitative results for molecular assays where

 10.1586/ERM.11.50 ISSN 1473-7159

standards are lacking [1–4]. Reporting guidelines and consensus testing for fragile X were developed as a result of poor performance by many laboratories in the 2002 and 2003 UK National External Quality Assessment Service fragile X PT schemes [5]. The American College of Medical Genetics (ACMG) assesses results from the College of American Pathologists PT surveys, and when a particular problem, such as poor agreement among laboratories occurs at a high frequency, disease-specific guidelines that address technical testing issues are developed [6]. ACMG guidelines for fragile X testing were written in response to poor performance on the College of American Pathologists fragile X proficiency survey [101].

The availability of reference materials and PT, the publication of guidance documents and the establishment of reference methods have lagged behind the rapid growth in molecular testing. The resources required to design and produce the required reference materials and guidance documents surpass those employed or volunteering to address those needs. These efforts are usually global and complex; drawing on experts from infectious diseases, genetics, molecular oncology and pathology, answering needs that span both qualitative and quantitative testing. In this article, we will attempt to describe some of the work that has established the materials and documents that are currently available. We will also explain the strategic approaches forming the foundation of the work.

Reference materials

Although the number of molecular diagnostic tests and their applications are numerous, all have a common requirement – the need for reference materials. While there has been progress on several fronts, there is a lack of established and globally accepted reference materials to serve as primary standards to compare performance characteristics and results of assays performed in different laboratories and/or using different methods. Reference material is defined as "material or substance, one or more of whose property values are sufficiently homogeneous and well established to be used for the calibration of a measuring system, the assessment of a measurement procedure, or for assigning values to materials" (International Organization for Standardization [ISO] 15195) [7]. There are a wide variety of possible reference materials that differ in the amount and type of characterization each has received. Some are designed as calibrators to help quantify an analyte, such as the amount of a certain virus in a clinical specimen, while others are qualitative and designed to standardize the detection of a particular DNA sequence, such as the Factor V Leiden mutation or the presence of a viral or bacterial pathogen. Other reference materials are used to develop and validate or verify the analytical performance of assays. Some are used for daily quality control to assure the performance of the assay and others are used for PT. The principal utility of reference materials has been to harmonize quantitative measurements, such as viral load measurements, or to establish the analytic sensitivity of qualitative assays. However, molecular testing, with the detection of complex sequences, mutations and biomarker patterns has escalated the need and importance for establishing qualitative reference materials with qualitative (nominal) properties.

Included in the category 'reference material' are certified reference materials (CRMs), standard reference materials (SRMs), calibrators, and characterized genomic nucleic acids. Quantitative CRMs and SRMs are characterized for composition and provide a primary way to trace quantitative measurements to the International System of Units (SI) without reference to a calibrator. CRMs are supplied with certificates that provide measurement results with associated uncertainties. SRMs are the versions of CRMs produced by the National Institute of Standards and Technology (NIST, US Department of Commerce). These primary reference materials may be used to establish traceability to the SI for secondary calibrants prepared by calibrant/reagent manufacturers or laboratories that produce their own calibrants. National Institute of Biological Standards and Control (NIBSC)/WHO International Standards (IS) are reference materials with consensus values assigned by an international interlaboratory study team or project. These are described as international convention calibrators by ISO 17511 [8]. The stated use for these materials is the same as that cited for CRMs. These two types of materials represent different approaches for reference material development and will be discussed in later sections of this article, using reference materials for CMV as an example.

Some CRMs and SRMs are certified for qualitative (nominal) properties such as DNA sequence. Other qualitative reference materials include characterized genomic DNA that contains previously characterized mutations or sequence variations, but with a lower level of confidence than provided by an SRM (Genetic Testing Reference Materials Coordination Program [GeT-RM] section). The role of qualitative reference materials in standardizing molecular testing is no less critical than reference materials carrying quantitative properties. Recognizing this importance, The Joint Commission on Traceability in Laboratory Medicine has published criteria on their website that addresses the evaluation of nominal properties for nucleic acid reference materials [102]. Nominally characterized reference materials are essential for developing and validating methods, daily quality control and as test materials for PT/EQA.

Reference materials provide a foundation for standardization, however, other important elements of standardization include ongoing assessment through PT/EQA and documentary standards. Documentary standards from the ISO, Clinical Laboratory Standards Institute (CLSI) and the European Committee for Standardization (CEN) provide guidance for the use of reference materials and methods, but are often difficult to use because they are written in general terms and do not usually address specific tests or disorders. Following the discussion of reference materials, the role of documents describing the characterization and use of reference materials, and the utilization of proficiency test programs for continued assessment of assay harmonization will be presented.

Reference materials with established quantitative properties

Manufacturers and testing laboratories seek globally established materials as the first calibration point in the development of new assays. There are very few SRMs or CRMs for molecular

methods, but those that exist are used to construct and validate calibrators produced by manufacturers or individual laboratories to establish a chain of traceability. ISO standard 17511 categorizes and provides strategies for the different types of materials. The predominant materials applicable to molecular testing include a few primary reference materials (CRMs and SRMs) and international convention calibrators (WHO/IS). These two categories differ with respect to how the materials are produced and characterized as well as how they might be used in laboratory assay standardization. The following two sections describe the scope of work and the processes behind two of the predominant organizations actively working in this area: NIST – producing SRMs; and NIBSC – producing WHO/IS. Both organizations are currently working on a CMV standard, therefore this example provides the opportunity to understand the separate strategies.

NIST SRMs

The National Institute of Standards and Technology is the national metrology institution for the USA with a mission to develop and apply technology, measurements and standards. NIST was approached by the clinical laboratory community, including the Association for Molecular Pathology, with requests to develop reference materials for molecular pathology to help fulfill the need for assay standardization.

The National Institute of Standards and Technology is developing SRMs for the quantification of viral disease. The first candidate SRM is CMV, to be followed by BK and Epstein–Barr virus (EBV). CMV and EBV are members of the family *Herpesviridae*, but represent different sub-families, while BK belongs to the *Polyomaviridae* family [9,10]. All three viruses are very common in the world population, often infecting individuals as children. The viruses remain latent for the life of the person, but may be reactivated when individuals become immunocompromised either through infection or during transplantation of organs or stem cells, where immunosuppressive treatment is necessary. Primary infections or viral reactivation can result in life-threatening illness and/or loss of the transplant. It is very important for physicians treating patients infected with these viruses to have an accurate measure of the viral load.

The material chosen by NIST to develop the candidate CMV SRM is the Towne strain, which has been cloned into a bacterial artificial chromosome (BAC) and provided to NIST [11]. Human CMV has a large genome and during *in vitro* culture conditions, both full and partial genomes may be packaged into virus particles [12]. A more consistent and complete CMV genome representation is derived from the propagation of the CMV BAC construct in *Escherichia coli* owing to the production of consistent DNA sequence by the bacterial DNA copy mechanism (stability) of BACs. The candidate SRM consists of CMV DNA in buffer at three different concentration levels and packaged in polytetrafluoroethylene tubes. Certification of the number of genome copies per sample volume, homogeneity of the SRM preparation and DNA stability studies are conducted on selected vials.

The efficiency of any PCR amplification reaction is dependent on exact sequence complementarity between the oligonucleotide primers, probe and the target viral DNA. Therefore, it is essential that the DNA sequence of the candidate reference material be substantiated and certified by NIST. The Sanger sequencing methodology has been used to characterize the CMV DNA used as targets for existing PCR assays. The sequenced regions of the Towne BAC clone selected for SRM development are exact matches for the Towne strain sequence in GenBank.

Alignments of primer and probe sequences of published PCR assays, with genomes of laboratory and clinical strains in the GenBank DNA database indicate a lot of DNA sequence variability for both laboratory strains and clinical isolates of CMV. For example, when 65 published primer and probe sequences were compared with the sequences of eight full genomes in GenBank, only 12 were perfect matches for all eight CMV genomes. To provide information on variation in sequence of the CMV genome, NIST has compiled a database, modeled on the long running DNA STRBase [103] for forensic DNA, which includes genomic sequences of published primers and probes with alignments to GenBank submissions of CMV sequences and other relevant information [104]. This database can be used as a resource for the molecular diagnostics community, particularly laboratories using laboratory-developed tests and calibrants.

Quantification of the candidate reference material in genome copies/volume is accomplished using digital PCR (D-PCR), the newest and most powerful tool for nucleic acid quantification. D-PCR methodology provides a way to count single DNA molecules, resulting in absolute quantification and traceability to the SI unit – the mole [13–15]. Validation of this approach is part of the process for certification of the candidate CMV SRM.

Digital PCR assays are independent of calibration. By contrast, real-time quantitative PCR is a relative measurement dependent on a calibration curve. Recently D-PCR instrument platforms have been developed with the capacity to perform massively parallel nano-sized assays on digital arrays with up to 9000 PCR reactions/array. The thousands of simultaneous assays contribute to the statistical accuracy of the final measurement. To provide absolute quantification, multiple experiments, utilizing an assay targeting a specific CMV gene, were conducted followed by a repeat of the process with five other assays targeting two other regions of the genome. The three components of the candidate SRM (dilutions of the same material) were separately quantified once it had been dispensed into vials.

There is work in progress to address the important issue of the ability of the NIST CMV standard to be used in a variety of assays (commutability). Quality Control for Molecular Diagnostics, an external quality assessment program for infectious disease testing, has included samples of the NIST CMV DNA with the lyophilized CMV samples in the 2009 and 2010 CMV challenges. The data fit to a Gaussian (normal) distribution, with most results within two log10, indicating that the NIST CMV standard gives similar results with different assay formats [Holden MJ, Unpublished data].

The protocol that has been developed to create the candidate CMV SRM, that is, certification of the DNA sequence and quantification in genome copies per volume using D-PCR, may serve as a model for development of future viral reference materials. A higher order CMV standard, such as the NIST SRM, is designed to be used to establish traceability, not as a daily calibrant. Manufacturers of calibrants, reagents and kits for measuring CMV viral load in clinical samples would establish traceability of the materials they develop to the NIST CMV standard. Traceability could similarly be established for laboratory-developed calibrants produced for use with laboratory-developed tests.

NIBSC/WHO International Standards

Biological reference materials (IS) are produced by NIBSC [105] and other collaborating centers under the auspices of the WHO. Reference preparations are available for a wide range of biological materials including therapeutics such as blood clotting factors, vaccines such as yellow fever [16], genetic markers, and virus preparations for standardizing molecular diagnosis [17–19]. The standards consist of a large number (3000–25,000) of ampoules with the same amount of material at a suitable level of activity for quantitation of the assays. The standard is prepared in a stably stored form, usually lyophilized, and monitored for quality, stability and uniformity. The standard is established by a collaborative study in which a few of the ampoules are distributed to invited participants with other samples of a similar type to assay by whatever method or methods they consider valid. If expressing the measurements relative to the reference material improves the agreement among laboratories and assays, the reference material is deemed suitable as a yard stick for the analyte in the various assays used. The study is reviewed by WHO and, if appropriate, the reference material is approved and made available.

The NIBSC/WHO rationale for using physical materials not traceable to the SI system as references is that absolute units such as grams are unable to capture the quantity of a complex biological activity or a complex analyte assayed by a complex biological method. For example, when a protein hormone is assayed, the critical feature is not the mass of protein present but the biological activity which may be measured by a number of methods, none of which encompasses all possible physiological effects *in vivo*. The active substance may also consist of mixtures of related molecules of differing specific activities in different proportions, such that the same aggregate activity can be composed of a vast number of different formulations with different masses. The mass is therefore not a meaningful expression of the biological activity which is the parameter of interest. Likewise, serum antibody preparations may have identical measurable activity, such as reaction in an ELISA, but the composition of an undeterminable possible number of combinations of different molecules with different individual binding properties may be present. In these circumstances, it is more rational to compare the unknown to a reference material with an arbitrary activity representing a consensus of the workers in the field rather than to express the results in mass or other SI units. The purity or nature of the reference or the biologically active material is irrelevant at this stage. In the case of a material that is fully characterized with potencies expressed in terms of mass (e.g., insulin), the determined mass is actually a surrogate for the real property of quantification which is biological activity. These are the principles of biological standardization.

Similar considerations might be applied to complex analytes such as nucleic acids, where the total amount of nucleic acid is not the essential matter, but the amount of nucleic acid of a particular microorganism or of a specific sequence is the significant measure. Using this justification, NIBSC asserts that molecular diagnostics assays are of a biological nature in the sense that they use enzymes and primers recognizing particular sequences and depend on extraction of nucleic acid from complex and variable matrices. They will therefore be influenced by uncontrollable variables in a way that physicochemical methods are not. Thus, expressing results in apparently absolute terms, such as detectable copy number, frequently gives a highly misleading appearance of analytical rigor.

Nonetheless, there is a need to quantify and compare the results obtained by individual laboratories. Sometimes this is crucial for decisions related to treatment: for example, the criterion for starting antiviral therapy for CMV can be a critical threshold of viral load. It is essential to ensure that the same conclusions are reached for patients with similar disease states so that the results in different laboratories and their clinical consequences may be compared. Comparisons can be made by the establishment of a biological reference material as similar to the analyte as possible, such as infected plasma in the case of hepatitis C or intact virus in the case of CMV.

Provided that the methods are comparable in their sensitivity, (e.g., reading low or high on all similar samples compared with the average), expression of the results in accordance with a common candidate reference material may greatly reduce the variation of measured values in the results. In a recent study which established an international reference for CMV DNA, 30 laboratories applied a total of 58 slightly different methods [Fryer J, Pers.Comm.]. The reported results varied by a range of more than two logs with a broad, flat distribution, yet expressing them as a ratio of the concentration of whole virus in the candidate reference material measured in the same laboratory produced much better agreement between different laboratories and a narrow unimodal distribution. The strain used was the Merlin strain but AD 169 was included and expressing the results in terms of the proposed reference improved the distribution in a similar way. A plasmid containing the complete Merlin strain genome did not improve agreement, suggesting that in the assays used, references based on the purified DNA and the intact virus were not commutable. Studies of this type are submitted to the Expert Committee for Biological Standardization of the WHO, which has a specific mandate from the UN for approval and establishment of the standard when scientifically justified. The unit is arbitrarily assigned by the Expert Committee for Biological Standardization and defined as a particular fraction of the contents of a single vial; there are strict criteria for the quality of the materials, including stability and consistency of the amount of material between vials [20].

One objection to this approach is that the stock of vials is finite and will therefore eventually require replacement. The replacement International Unit, defined as a fraction of the content of the activity in a vial of the new reference must be as close to the previous one as possible, but there is uncertainty associated with the calibration. Because the unit is defined as a fraction of the vial contents the establishment of the second standard means that the first unit ceases to exist and the second unit will be slightly different from the first; the difference should be undetectable in the context of the assays performed, which rarely have a precision or accuracy approaching that of physico-chemical methods. The finite stock of the reference materials implies that they are intended for calibration of secondary reference materials.

Summary of approaches to quantitative reference materials

While there is agreement about the intended use of these reference materials (as calibrants for the production and maintenance of secondary standards in clinical and test development laboratories), there are philosophical differences between the approaches taken by NIST and NIBSC in their development and characterization of reference materials. NIBSC has included nucleic acid assays for viral load in the category of biological activity measurement. NIST interprets such assays as the detection of DNA sequences specific to a virus' genome, not a biological activity assay. The differences in approach to standard development and the two types of standard materials that result from these differences are summarized in Table 1.

There is currently no reference method to quantify nucleic acids. Thus, there is a potential for the value of replacement biological standards to drift as new ones are adopted. A process could be developed to harmonize a biologic standard with a NIST SRM and then using that relationship to aid in the value assignment of replacement biological standards; especially when testing methods change. Instead of only relating a potential replacement biological standard to the existing one, the SRM that was established with the first biological standard is also tested. This allows the value assignment of the new replacement biological standard to be compared with the existing biological standard (current practice) using the SRM which represents the original relationship between the two.

The two CMV standards described here, the IS from NIBSC and the SRM from NIST, provide the first opportunity to test this strategy. When the acceptable method for determining pathogen 'load' is nucleic acid quantification, it will be important to continue to develop strategies to create new materials and to assure harmonization and continuity with those that already exist.

Reference materials with nominal properties

GeT-RM: development of characterized genomic DNA reference materials for genetic testing

Reference and quality control (QC) materials are essential for many aspects of genetic testing. These materials, which are tested alongside patient samples, allow the laboratories to detect errors due to test system failure or operator error. In addition, reference materials are needed for test development and validation, lot-testing of new reagent batches and for PT/EQA.

Over 1900 genetic tests are currently offered in clinical laboratories [106], however, for the vast majority of these tests, no publicly available characterized reference or QC materials are available. SRM, CRM and WHO reference materials have been produced for only a few disorders (fragile X syndrome, prothrombin, Prader–Willi syndrome, Angelman syndrome, Huntington's disease and factor V). In the absence of publicly available reference materials, laboratories must seek out sources of materials from the community. Often, DNA derived from leftover patient specimens, which is not easily available or renewable, is used as a control material. Laboratories also utilize synthetic DNA or DNA isolated from cell lines. All of these materials must be validated by the laboratory prior to use as QC or reference materials.

The CDC has been involved since 1995 in efforts to develop appropriate and well characterized reference materials for use by the genetics community. In 2004, the GeT-RM was established at the CDC in partnership with the genetics community. The goal of this program is to coordinate a self-sustaining community process to improve the availability of characterized genomic DNA materials for quality control, PT, test development/validation and research. The GeT-RM also facilitates information exchange between users and providers of reference materials. Although the GeT-RM program is coordinated by the CDC, all of the actual work, including decisions about reference material priorities, specimen collection, material development and characterization occurs through voluntary collaborations with laboratories in the genetics community. Cell lines with confirmed genotypes are considered the preferred type of control for DNA-based genetic testing as they most closely resemble an actual patient specimen. Thus, the GeT-RM's efforts focus on this material type.

The GeT-RM program has recently characterized more than 200 cell line-based genomic DNA reference materials for a number of genetic disorders, including: fragile X syndrome [21], disorders on the Ashkenazi Jewish Panel (Bloom syndrome, Canavan disease, Fanconi anemia, familial dysautonomia,

Table 1. Differences in approach to the development of reference materials for quantitative infectious disease.

NIST	NIBSC
Characterized material – pure viral DNA with sequence verified	Uncharacterized intact virus or clinical material
Absolute quantification, independent of assay	Consensus evaluation, assay-dependent value
Value assignment in genome copies/volume with reported uncertainties	Value assignment in International Units specific to that lot of material, no uncertainty reported

NIBSC: National Institute for Biological Standards and Control; NIST: National Institute of Standards and Technology.

Gaucher disease, mucolipidosis IV, Neimann–Pick disease and Tay–Sachs disease) [22], cystic fibrosis [23], Huntington's disease [24], methylenetetrahydrofolate reductase-related homocysteinemia, α1-antitrypsin deficiency, multiple endocrine neoplasia and BRCA1- and BRCA2-related cancers [25]. Genomic DNA material was tested in between three and ten clinical genetic laboratories for each of these disorders using a variety of genetic assays, including DNA sequence analysis. These materials are publicly available from the Coriell Cell Repositories (NJ, USA). The GeT-RM has recently completed characterization studies of genomic DNA reference materials for Duchenne muscular dystrophy [26], as well as a large-scale study of DNA from 107 cell lines for a number of polymorphisms in 20, mostly pharmacogenetic, loci [27]. A large project to develop characterized genomic DNA reference materials for molecular cytogenetics is currently underway.

The GeT-RM has primarily focused its efforts on DNA-based testing for inherited genetic disorders. However, there is a similar lack of reference materials for other areas of genetic testing, including molecular oncology, molecular infectious disease testing and biochemical genetic testing. Mechanisms to address reference material needs for these areas are also being considered.

The GeT-RM website provides a comprehensive source of molecular genetics reference material information to the genetic testing community [107]. The website is grouped into three subject areas; inherited genetic diseases and pharmacogenetics, molecular oncology and infectious disease. Information about available reference materials, including applicable characterization studies and results are provided. The website also features comprehensive searchable databases of commercially available reference materials for both molecular oncology and infectious disease and general information about reference materials, including pertinent research articles, a list of reference material sources (including manufacturers and repositories) and a list of websites with relevant guidance documents.

NIST SRMs with nominal properties

The National Institute of Standards and Technology has also produced two SRMs for clinical genetics. SRM 2399 is the Fragile X Human DNA Triplet Repeat Standard. Fragile X syndrome is the most common inherited form of mental retardation. This disorder is caused by an expansion of a triplet repeat (CGG) in the *FMR1* gene on the X chromosome [28]. SRM 2399 consists of nine components, eight of which have a certified number of trinucleotide repeats plus a ninth sample with a noncertified information value. These components consist of PCR products amplified from genomic DNA of cell lines with a variety of triplet repeat lengths. The PCR products that constitute this SRM have been sequenced using Sanger sequencing and the number of repeats range from 20 to 118. It is critical that the number of trinucleotide repeats are correctly determined for the clinical utility of testing patient specimens. The full mutation (>200 repeats) is associated with severe developmental delay or mental retardation. Individuals that carry repeat expansions within the premutation category (55–200 repeats) may develop late-onset neurological symptoms (fragile X-associated tremor and ataxia syndrome) or premature ovarian failure. Females with premutation risk passing an expanded allele to their offspring, and this risk rises with larger repeat lengths [108]. The SRM covers the range for normal (5–44 repeats), intermediate (45–54) and premutation (55–200) [108]. There is no SRM component that covers the full mutation (>200 repeats).

A second SRM (SRM 2393) for clinical genetics has recently become available and is designed to ensure the accuracy of test results and method validation for Huntington's disease. Like fragile X syndrome, Huntington's disease is also caused by a trinucleotide repeat expansion which results in a degenerative brain disorder [29]. The repeats (CAG) are found in the genetic locus, *HTT* on chromosome 4 (4p16.3) [109]. The severity of the symptoms in affected individuals with expanded Huntington alleles is dependent on the CAG repeat length [30]. Individuals with 35 or fewer repeats do not develop the disorder, alleles with 36–39 repeats have reduced penetrance and alleles with 40 or more repeats are fully penetrant [31]. The components of SRM 2393 consist of genomic DNA from cell lines derived from samples from patients with Huntington's disease. The certified values in the SRM components cover the defined range of repeat values [31] for Huntington's disease alleles; normal (<26 CAG repeats), mutable normal (27–35), Huntington's disease allele with reduced penetrance (36–39) and Huntington's disease allele (≥40) [30]. The values for SRM 2393 were established by genotyping and DNA Sanger sequencing [110].

The National Institute of Standards and Technology has been involved with the External RNA Controls Consortium (ERCC) formed in 2003. The ERCC was founded for the purpose of developing traceable external RNA controls that would be useful for evaluating data generated by microarray and quantitative reverse-transcriptase PCR experiments. Enthusiastic early adoption of DNA microarrays was fraught with unpredictable performance and uncertain results [32]. More than 90 organizations representing academia, industry and government have participated in this project to develop a plasmid DNA library, the candidate NIST SRM 2374 DNA sequence library for external RNA controls. The library consists of plasmids with 96 different DNA fragment inserts to direct the expression of transcript RNA controls [33]. The certified property of SRM 2374 will be the DNA sequences of the 96 control constructs determined by the NIST and other institutions. RNA controls will be generated by manufacturers and core facilities. The RNA controls derived from the templates in the SRM are intended for use as external, or 'spike-in', controls to support confidence and provide measurement assurance in gene-expression assays, regardless of instrument platform and assay type [34].

WHO International Genetic Reference Panel

A genetic reference panel for fragile X syndrome has been developed and established by the WHO [35]. The panel consists of genomic DNA isolated from immortalized lymphoblastoid cell lines. The cell lines were immortalized using EBV transformation to provide a stable source of genomic DNA for the future. Materials were chosen from male and female individuals with normal, premutation and full mutation alleles. This reference

panel differs from the NIST SRM discussed previously, which is composed of PCR products amplified from genomic DNA. Table 2 provides information regarding reference materials and the sources for them as described previously, including some commercial reference materials, controls and proficiency panels. The US FDA provides information on various materials that have passed their clearance process.

Documentary standards

The establishment, maintenance and distribution of reference materials provide the critical foundation for comparable and commutable molecular assays. However, availability of the reference materials alone does not guarantee improved harmonization. Approaches for their implementation in test development and validation should be uniform and harmonized. These approaches are provided as documentary standards, guidelines, consensus documents and reference method publications. There are many standards and guidelines issued by numerous organizations. Test developers are challenged to find them, evaluate them and identify those with the most relevance to their application(s). Standards and guideline documents are generally produced by standard document organizations such as the ISO and CLSI; regulatory organizations such as the FDA and the EU; and professional organizations such as the ACMG, the National Academy for Clinical Biochemistry and the Association for Molecular Pathology. Procedural method publications are often written by professional groups or individual experts (Table 3 gives a list of principal organizations that produce standards and guidelines relevant to molecular diagnostics standardization). There are a growing number of standards, guidelines and procedures addressing all aspects of medical laboratory test development, implementation and interpretation in clinical molecular diagnostics. Describing each of their attributes and applications is beyond the current scope of this article. The primary focus of this section will be the context and source of these documents, with some examples specific to their use in establishing and using standards and reference materials for molecular diagnostics.

Finding a relevant document for use with standard materials depends on the intended purpose of the test and the reference materials. Documents outlining the framework for the establishment and characterization of standard materials and strategies for employing them are frequently produced by organizations such as ISO, CLSI, the CEN and national regulatory bodies. Those publications that define actual methods and procedures for use may be found through the organizations supplying the materials, or may be produced as peer-reviewed publications by expert groups or individuals. Some organizations such as CLSI span strategic, general and specific methodological approaches in their document libraries. Some regulatory agencies adopt specific voluntary standards and guidelines published by consensus organizations such as ISO, CEN and CLSI instead of producing their own.

Importantly, the relevance of documents to molecular methods may not be readily apparent. This is particularly true of the strategic framework documents: the ISO documents, CEN documents and CLSI documents that address evaluation protocols or chemistry specialties. Medical laboratories have a long tradition of quality practices and operations, which includes standardization, verification and validation. Framework and strategic general documents and guideline documents for some technical areas of the clinical laboratory provide approaches that may be broadly adaptable. Sometimes the translation to molecular applications is seamless, other times there are gaps.

Several regulatory and voluntary consensus documents state that clinical laboratory assays must be traceable to reference materials, whether they are produced by manufacturers or developed within medical laboratories (European Standard 98/79/EC; ISO 17511: 2003; ISO 15194: 2009) [8,36,37]. Even controls and proficiency test materials must demonstrate traceability to established standard reference materials where applicable (FDA 1999; ISO 17511: 2003; ISO 17043: 2010) [8,38,111]. The standard, ISO 17511: 2003 [8] lays out the foundation and nomenclature of metrological traceability in laboratory medicine using hierarchical orders of reference materials and procedures.

Table 2. Sources for reference materials or information about available reference materials.

Institution	Types of reference materials	Infectious disease	Heritable genetics	Molecular oncology	Pharmacogenomic	Ref.
NIBSC, WHO	International Standards	✓	✓	✓		[115]
NIST, Department of Commerce, National Metrology Institute USA	Standard reference materials	✓	✓			[116]
IRMM, Joint Research Center, National Metrology Institute EU	Certified reference materials		✓			[117]
US FDA, HHS USA	FDA-cleared reference materials and controls (instrument platforms and assays)	✓	✓		✓	[118]
GeT-RM, Center for Disease Control, HHS USA	Information resource GeT-RM program studies	✓	✓	✓	✓	[107]

GeT-RM: Genetic Testing Reference Materials Coordination Program; HSS: Department of Health and Human Services; IRMM: Institute for Reference Materials and Measurements; NIBSC: National Institute for Biological Standards and Control; NIST: National Institute of Standards and Technology.

Table 3. Example of principal organizations producing practice standards and guideline documents relevant to molecular diagnostic standardization.

Organization	Description & types of documents produced
Regulatory	
EU	Produce regulations that become binding in member states
US FDA†	Produce regulations and guidance documents that explain their interpretation of and/or guide compliance to US regulations
Voluntary	
CEN†	Provides a standardization framework to prepare voluntary standards supporting the development of a single European market for goods and service A European standard automatically becomes a national standard in the member countries
ISO†	A network of national standards institutes from 163 member countries. The standards written are voluntary but can be adopted by regulatory agencies
CLSI†	A global voluntary consensus organization. Provides a framework to produce voluntary global standards and guidelines with a primary focus in clinical laboratory science. Standards and guidelines from CLSI can be adopted by regulatory agencies
ILAC†	An organization of accrediting bodies for testing and calibration laboratories. Produces general documents to assist in the accreditation process
ASTM International	Develops international voluntary consensus standards documents in many areas. In clinical molecular science, have documents on focused molecular methods

†These organizations have documents that address the use of standard reference materials in diagnostic assay development and implementation.
ASTM: American Society for Testing and Materials; CEN: Committee for European Standardization; CLSI: Clinical and Laboratory Standards Institute; ILAC: International Laboratory Accreditation Cooperation; ISO: International Organization for Standardization.

It stratifies reference materials and reference procedures into these orders based on their connection to the base units in the SI units and absoluteness of the reference procedure to assign value. For example, the highest order reference, a primary standard, is one that is directly calibrated to the appropriate SI unit. It is assigned by an internationally recognized primary reference procedure, traceable to the appropriate SI unit of measurement, without reference to a calibrator. The measurement procedure must be performed in a qualified measurement laboratory (qualified according to ISO 17025:2005) [39] and the uncertainty of the measurement procedure must be quantifiable. For materials that are not metrologically traceable to SI, the hierarchy follows combinations based on the availability of reference methods, internationally accepted procedures, internationally recognized calibrators and/or value-assignment protocols. ISO 17511: 2003 [8] provides the scaffolding to understand where the currently available molecular standards, reference materials and reference procedures rank in the hierarchy of metrology. Many of the current molecular reference standards, particularly the WHO IS for infectious disease agents, are produced and assigned by global collaborative studies directed by NIBSC. The development and selection of the materials, including the study method and findings are usually published [40,41]. Access to standard documents describing these frameworks and approaches facilitates communication within the clinical genetic testing community specialty and within the broader area of clinical laboratory medicine and regulatory oversight. This enhances the discussions of strategy, gaps and implementation using a common nomenclature with a common understanding of risks and benefits.

International Organization for Standardization document 15194:2009 describes the characterization of reference materials and calibrators, and introduces the topic of commutability for reference materials [37]. C53, a CLSI guideline, describes usable approaches to evaluate commutability, in addition to strategies for characterizing the homogeneity, stability and traceability of reference materials [42]. For a more detailed illustration of an actual application, a publication describing the assessment of commutability for a specific CMV calibrator to two laboratory-developed assays is described by Caliendo *et al.* [43]. The CLSI document and the paper can form the basis of design for further studies.

In addition to knowing how the molecular standards and reference materials are established, it is critical to have common strategies for employing them in assay development. Table 4 provides a limited list of documents that address approaches, practices and issues to consider for developing tests with the currently available types of standards and reference materials and for specific areas of molecular testing. Most of these documents address general principles in laboratory test development, thus highlighting the scarcity of documents specifically addressing molecular testing. Professionals in molecular diagnostics frequently adapt elements of these general approaches to their own test development applications. One example is the companion development of standard reference materials with specific guidance documents describing strategies and methods for their uses. In the clinical chemistry area, a CLSI document, C39 – A Designated Comparison Method for the Measurement of Ionized Calcium in Serum; Approved Standard [44] and NIST SRM 956a provide a model. C39 addresses the assignment of ionized calcium concentrations to NIST SRM 956a. It describes the materials and methods used, and the results and conclusions of an interlaboratory study used in the assignment. Although nucleic acid molecules are not as elemental as calcium, the mechanics of these complimentary standard reference materials/standard documents may be

Table 4. Table of documents with particular application to the use of standards and reference materials that could be applied to molecular method strategies.

Document	Description	Ref.
Development & establishment of standards & reference materials		
ISO 17511: 2003 *In vitro* diagnostic medical devices – Measurement of quantities in biological samples – metrological traceability of values assigned to calibrators and control materials	Sets the framework of the metrological hierarchy of standards, reference materials and calibrators, and specifies the strategies and requirements for traceability of calibrators and control materials – including external quality assessment samples	[8]
ISO 15194: 2009 *In vitro* diagnostic medical devices – Measurement of quantities in samples of biological origin – requirements for certified reference materials and the content of supporting documentation	Describes the process for producing and assigning value to certified reference materials. Introduces the concept of commutability	[37]
WHO (2004) Characterization and establishment of international and other biological reference standards	Describes the process for establishing the WHO International Standards	[41]
CLSI X05-R. Metrological traceability and its implementation; a report	CLSI–IFCC joint project report that describes the framework of traceability documents and provides guidance for establishing and reporting metrological traceability	[56]
Use of standards & reference materials in assay development, verification & validation		
ILAC G9 Guidelines for the selection and use of reference materials	Establishes a framework by which laboratories seeking accreditation, and technical assessors evaluating them, will be able to propose and evaluate standards and reference materials relevant to their specific needs	[57]
CLSI C53 Characterization and quantification of commutable reference materials for laboratory medicine	Guidance in the production and characterization of commutable reference materials and provides information for their proper use in calibration and trueness assessment of *in vitro* diagnostic medical devices	[42]
CLSI MM6 Quantitative molecular methods for infectious diseases	Section 7 provides a discussion on the types of standards and reference materials available and their role in the development, validation and verification of assays	[58]
CLSI MM10-A Genotyping for infectious diseases: identification and characterization	Section 7 provides a discussion on the use of different types of standards and reference materials in genotyping assays for infectious diseases	[59]
CLSI MM16 The use of external RNA controls in gene-expression assays	Describes the use of external RNA controls in the development and assessment of microarray and QPCR tests	[45]
CLSI MM17 Verification and validation of multiplex nucleic acid assays	Discusses strategies for using human DNA, whole genome or synthetic standards, and reference materials in section 7	[52]

CLSI: Clinical and Laboratory Standards Institute; IFCC: International Federation of Clinical Chemistry; ILAC: International Laboratory Accreditation Cooperation; ISO: International Organization for Standardization.

applicable. In the molecular area, there is a similar companion pair with MM16 – Use of External RNA Controls in Gene Expression Assays [45] and a library of 96 RNA controls developed by the ERCC and NIST [33]. MM16 describes strategies for the use of the ERCC RNA controls, or any similarly designed substances in microarray and quantitative real time PCR-based gene-expression experiments.

Molecular methods have few reference materials and no consensus reference methods. This section has described how existing documentary standards can, and should, be used as the basis for development in this area. The area of laboratory practice guidelines in molecular diagnostics for general quality management, test implementation and disease specific applications, is more developed (Table 5). Further work to develop these and other practice guidelines must continue as the field grows and diversifies. Even more critical is the need for the establishment and harmonization of standard reference materials with companion documents. These materials and documents must be developed in accordance with accepted organizations and processes.

Proficiency testing: ongoing standardization & assessment

Proficiency testing (EQA) has become engrained in clinical laboratory processes since the late 1940s [46]. Though not their primary purpose, formal and informal PT schemes have also served as indicators of the relative performance accuracy of

Table 5. Sources for documents addressing molecular diagnostic test implementation and practice for laboratories.

Supporting group or agency	Description of resources	Ref.
ACMG	Publishes general and disease-specific laboratory standards and guidelines, as well as clinical practice guidelines for medical genetics	[119]
AMP	Professional organization that produces clinical practice guidelines for general and specific molecular laboratory practices	[120]
CDC	Produces some guidelines on clinical laboratory practice in some areas, particularly one guideline for medical genetic testing [60]	[121]
CAP	Provides checklists for laboratories requesting accreditation Special checklist for molecular pathology Also writes articles in molecular test method validation	[122]
CLSI	**Evaluation protocol documents:** Documents providing best practices in clinical laboratory test method evaluation: qualitative, qualitative, estimations of bias **Molecular methods documents:** Provides best practice guidelines specifically in the areas of molecular methods: infectious diseases, genetics and hematopathology	[123]
EuroGentest	Provides links to several general and procedural/technical best practice documents in genetic testing: from publications and organizations	[124]

ACMG: American College of Medical Genetics; AMP: Association for Molecular Pathology; CAP: College of American Pathologists; CDC: Centers for Disease Control and Prevention; CLSI: Clinical and Laboratory Standards Institute.

various methods and the variability in measurement between clinical laboratories. During the rapid development of quantitative molecular tests for viral diseases, indicators of the realistic interlaboratory alignment of these assays were provided by distribution of coded panels among laboratories, and later, formal PT surveys. It was observed that the agreement for some assays and methods was not optimal. However, with the establishment of quantitative international standards (HIV, hepatitis C virus) and the continued assessment of laboratory performance, improvement was demonstrated [47–50]. Quantitative molecular infectious disease tests that have no established standards continue to demonstrate problems with interlaboratory alignment [1,2,4]. The establishment of standards and PT materials calibrated to those standards, can help to improve agreement between assays when used together. It has been recommended that proficiency test panels, in addition to ongoing quality control material, be calibrated to standards when they are available [51].

Since the mid-1990s, more PT programs for molecular genetic tests for heritable genetics, oncology and pharmacogenetics analytes have been developed. Owing to the large number of new tests that are introduced each year, the demand for PT schemes is expanding more rapidly than formal PT programs might accommodate. In addition, many of these new tests are only offered in one or a few laboratories, making formal PT economically unfeasible for traditional programs. To bridge these gaps, professional organizations and PT providers have begun to act as facilitators of formal sample exchanges [112]. In addition, methodology-based PT has been explored as a means to assess areas of laboratory testing where many molecular tests utilize the same method, such as DNA sequence analysis [5,52]. CLSI guidelines also provide recommendations for alternative strategies [51,52].

Some PT programs publish their results online (American Proficiency Institute and NY state [113,114]) or as scientific articles [53–55]. Presentation of the results of PT assists not only the participants, but also the global community to assess the relative standardization of practicing laboratories. The molecular testing community has actively promoted the calibration of PT panels to recognized standard materials where possible, and the adoption of alternative assessment methods. Interlaboratory comparisons that are facilitated by PT program providers have been a source of information supporting this advocacy. Table 6 provides a list of some of the current PT organizations with offerings in different areas of molecular testing. The areas of service might seem comprehensive, but some of the organizations only offer PT for one or two test offerings in the categories listed; more programs are needed to encompass growing test menus of clinical laboratories. A detailed discussion of these programs, their scope of service and offerings is beyond the scope of this article. PT strategies to evaluate the performance of new technologies are urgently needed.

We have discussed three separate topics in this article; reference materials, documentary standards and PT. Figure 1 presents a synthesis of this article and the interrelationship between the three components. These are tools that might be used effectively to increase accuracy and harmonization of results of molecular diagnostic testing.

Expert commentary

The field of molecular diagnostics and testing is evolving rapidly. Developments in basic and clinical research provide new information on the molecular basis of disease. Nucleic acids that are considered appropriate testing targets may be replaced with new targets. Furthermore, new technology replaces established methods and approaches, such as PCR based detection versus viral culture. Some of the new technologies, such as Next-Gen sequencing, provide a wealth of information. The mining of relevant information is in the early stages but promises to provide the basis of individualized medicine. How good is that information? What is an appropriate standard for technology

Table 6. Resources for proficiency testing for molecular assays.

Organization	Infectious diseases	Heritable genetics	Molecular oncology	Pharmacogenomics	Technique based	Facilitate sample exchanges	Ref.
CAP	×	×	×	×		×	[122]
QCMD	×						[125]
New York State CLEP Program			×				[126]
EMQN		×			×		[127]
UK NEQAS		×	×		×		[128]
German Society for Clinical Chemistry and Laboratory Medicine	×	×			×		[129]

CAP: College of American Pathologists; CLEP: Clinical Laboratory Evaluation Program; EMQN: European Molecular Genetics Quality Network; NEQAS: National External Quality Assessment Scheme; QCMD: Quality Control for Molecular Diagnostics.

such as Next-Gen sequencing? Providing standards that remain relevant for molecular testing is a large challenge. Not only are there no or limited availability of standards for many important testing targets, but the current ones could be rendered obsolete for some of the reasons described above. Producing standards is expensive, time consuming and often difficult owing to the complex nature of the measurands. They represent a significant investment. Standards are even more important when considering the many available tests for a given target, both commercial and unique tests developed in individual laboratories. The plethora of tests also highlights the need for PT programs, and participation in these programs has steadily increased. This is a critical tool for ensuring the accuracy of a given test and the harmonization of testing results between laboratories. Communication between investigators and clinicians, producers of standards, controls, molecular testing kits and reagents, as well as regulatory bodies, is critical to the future of quality testing necessary for patient care.

Five-year view

Molecular genetic testing is currently being used in many areas of laboratory testing. An increasing number of tests that had previously utilized chemical or immunological methods, including blood and HLA typing, are now incorporating molecular methods. This trend is expected to increase in the next few years. At the same time, new technologies that can analyze or sequence the entire genome in a short period of time are being incorporated into laboratory testing. This will allow diagnosis of genetic conditions, analysis of predisposition to disease and determination of phenotypes such as pharmacogenetic profiles or blood types throughout a patient's lifetime using data generated from a single test performed once.

In the past, concerns centered on developing reference materials for a particular genetic test, such as cystic fibrosis or fragile X syndrome, which typically involved only a single gene. The analysis of whole genomes will require a very different approach to reference material development and use of reference materials for quality assurance. Involvement of the diagnostics community in providing input and participating in projects related to reference material use and development could lead to greater harmonization in testing. We have discussed two examples of how this has worked well – GeT-RM and the ERCC.

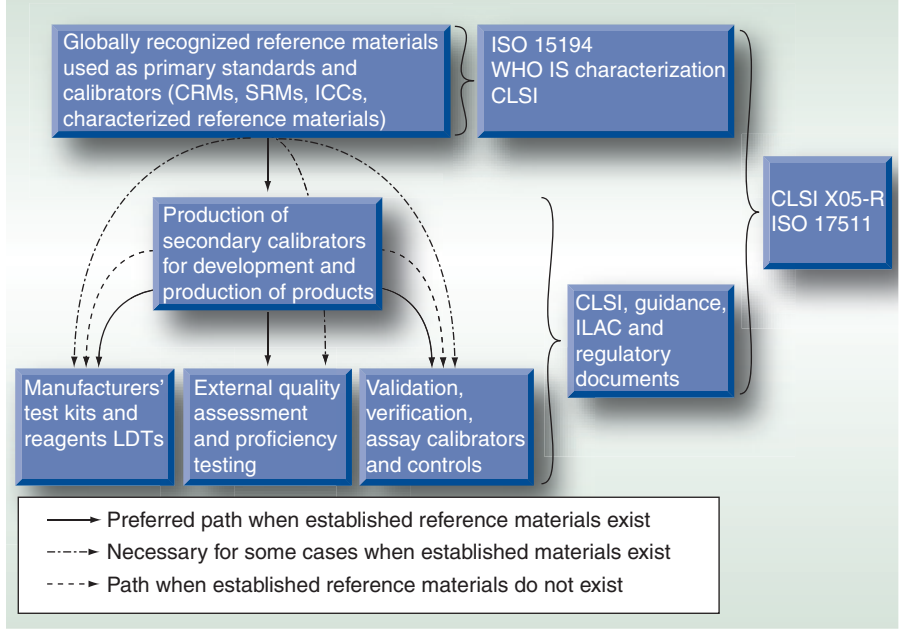

Figure 1. The inter-relationship and influence of reference materials and standard-guidance documents on assay development and quality assessment.
CLSI: Clinical and Laboratory Standards Institute; CRM: Certified reference material; ICC: International convention calibraters; ILAC: International Laboratory Accredited Cooperation; IS: International standards; ISO: International Organization for Standardization; LDT: Laboratory-developed test; SRM: Standard reference material.

The GeT-RM is currently working on the development of characterized genomic DNA reference materials for two types of whole genome tests: cytogenetic microarray analysis and whole genome sequence analysis. Using DNA generated from Coriell cell lines, GeT-RM will create two characterized genomic DNA RM panels. The clinical panel will consist of approximately 100 genomic DNA samples with specific chromosomal abnormalities typically detected in clinical cytogenetics laboratories. These include microdeletions and microduplications, subtelomeric abnormalities, loss of heterozygosity and uniparental disomy and other genetic variations. The probe evaluation panel will consist of approximately 250 genomic DNA samples containing large abnormalities that collectively encompass most of the genome by both deletions and duplications to evaluate the performance of assay probes utilized in any cytogenetic array. Following cell line selection, DNA from each will be characterized using a variety of commercially available cytogenetic microarray analysis platforms to confirm the copy number status of each genomic imbalance and to assess their suitability as RMs. New cell lines from recently described novel micro-deletion/duplication syndromes will also be established. Required cell lines that currently exist outside of Coriell will also be used for this project. The GeT-RM is also considering approaches to develop characterized genomic DNA for whole genome sequencing.

There is a third community effort that is in the planning stage. In October 2010 the American Association for Clinical Chemistry held a meeting entitled 'Improving Clinical Laboratory Testing Through Harmonization: an International Forum'. The purpose of the conference was to propose a framework for making decisions and developing protocols for harmonization in the measurement of analytes where there may be no reference method or reference material. This forum was not intended to discuss or make decisions on developing reference materials or harmonizing methods for detection of specific analytes (small molecules, proteins and nucleic acids), though they were used to illustrate problems and issues. The framework of the meeting was generic and was intended to be the beginning of an ongoing process. A structure was formulated at the meeting, and will be refined and used for harmonization of specific analytes. Eventually, this design process will be conducted online.

Finally, the next 5 years should see increased cooperation in the development of reference materials between organizations such as NIBSC/WHO laboratories and national metrology institutions, such as NIST. The place to start is with the CMV reference materials relating the values of the international units for the WHO IS and the NIST SRM genome copies per ml.

Standardization in molecular diagnostics carries the challenge that new technologies have struggled with in the past and will continue to face reinvention versus invention. Strategies for the establishment of standard materials exist in several global and regional organizations, but there is a need to recognize where they could be adapted to harmonize or complement each other. To meet the needs of new technologies, our current understanding of standard reference materials may have to expand to new consensus indicators of specimen or measurand integrity.

Proficiency testing programs are imbedded in the laboratory quality process, however, new approaches that can quickly adapt to the rapid addition of new measurands and techniques need to be considered. In addition, new standards documents should be aligned with globally accepted foundation documents, such as ISO documents and high priority should be given to producing documents where there are gaps. In addition to producing more standard resource material for existing molecular genetic tests, work in this area, such as taking inventory, indentifying gaps and prioritizing work forward, will pave the way for the standardization of new tests and new technologies in the future.

Disclosure

The findings and conclusions in this presentation are those of the authors and do not necessarily represent the views of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Key issues

- The rapid development of molecular methods for the detection and quantification in clinical diagnostics for infectious disease, oncology and heritable diseases increases the need for nucleic acid-based reference materials.
- Reference materials can take different forms and serve different roles: primary (or higher order) for the development of traceable quantitative calibrants, and secondary standards for method validation or daily controls.
- Reference materials can be certified for quantity or for qualitative (nominal) properties such as DNA sequence.
- The use of reference materials, appropriate documentary standards and guidelines, and proficiency testing can lead to greater accuracy of results and harmonization of testing among laboratories.
- There are large gaps between what is available and what is needed. Creative communities of stakeholders are functioning to help fill the gap, but more is needed.
- Development of metrological approaches for complex measurands, such as the whole human genome, will need to be considered in the near future.

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