Standardization of Nucleic Acid Tests

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Published in:
Journal of Clinical Microbiology

DOI:
10.1128/JCM.01056-18

Publication date:
2019

Standardization of nucleic acid amplification tests: the approach of the World Health Organization

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ABSTRACT

The first World Health Organization (WHO) International Standards (ISs) for nucleic acid amplification techniques (NAT or NAAT) were established two decades ago with the initial focus on blood screening for three major viral targets - hepatitis C virus, hepatitis B virus and human immunodeficiency virus type 1. These reference materials have subsequently found utility in the diagnosis and monitoring of a wide range of infectious diseases in clinical microbiology laboratories worldwide. WHO collaborating centers develop ISs and coordinate international studies for their evaluation. The WHO Expert Committee on Biological Standardization is responsible for the endorsement of new standardization projects as well as establishment of new and replacement ISs. Potencies of ISs are defined in “international units” (IU), and the reporting in IU by assays calibrated with an IS (or secondary standards traceable to the IS) facilitates comparability of results between different assays and determination of assay parameters such as analytical sensitivities.

INTRODUCTION

Nucleic acid amplification technology (NAT or NAAT) has become a staple in both the clinical microbiology laboratory and in blood screening centers for the detection of microbial pathogens, particularly viruses. This was not the case more than two decades ago with the transmission of hepatitis B/C viruses (HBV and HCV) and human immunodeficiency virus type 1 (HIV-1) to recipients of therapeutic plasma derivatives or blood components, when it was realized that closing the serological window using NAT testing improved blood safety. In the following years, considerable effort was invested in the implementation of NAT screening for blood and plasma donors and introducing this technology for diagnostic testing in clinical microbiology laboratories using both commercial as well as laboratory developed tests (LDTs). However, assay sensitivities and specificities varied widely between laboratories, contamination by amplicons was problematic and assays lacked standardization. During this

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time, the World Health Organization (WHO), as the global institution for setting standards for health systems, was requested to establish internationally accepted reference materials, e.g. International Standards (ISs), for NAT assays. The ISs are measurement standards with a defined concentration of a specific analyte that enable the comparison of results between different assays and different laboratories. These reference materials were initially prepared from viremic plasma donations (reflecting the type of sample being tested) and subsequently freeze dried. The complex nature of donor and clinical samples, such as plasma or sera, means that nucleic acid measurement of a specific pathogen cannot be determined by physico-chemical methods. Before nucleic acid concentrations can be determined, samples must be extracted and undergo *in vitro* amplification and detection; therefore results cannot simply be reported in International System of Units (SI)-related units such as kilograms or moles. For WHO ISs representing complex biological materials, the WHO took the approach of adopting the International Unit (IU); the IU has been used to define potencies of all ISs for NAT-based assays.

In this review, we discuss the steps involved in prioritization and in the preparation and characterization of WHO ISs, their establishment, replacement and realization of their value in harmonizing results between different assays and different laboratories.

SETTING PRIORITIES FOR NAT STANDARDIZATION

An international working group Standardization of Genomic Amplification Techniques (SoGAT) was established in 1995, on behalf of the WHO, which has since been coordinated by the National Institute for Biological Standards and Control (NIBSC; United Kingdom). Initially, the focus was to standardize NAT assays for blood-borne pathogens important in the field of blood safety; however, standardization was also essential in the diagnosis and monitoring of infectious diseases in the clinical setting. WHO ISs for pathogens such as HCV,

HBV and HIV-1 have been widely used in microbiology laboratories as well and new standards have been prepared for increasing numbers of clinically important pathogens. The first WHO IS for NAT assays established in 1997 was HCV (1), this was followed by hepatitis B virus (HBV) and HIV-1 in 1999 (2, 3). Subsequently, ISs have been established for other blood-borne viruses including parvovirus B19 (B19V), hepatitis A virus (HAV), HIV-2, hepatitis E virus (HEV) and hepatitis D virus (HDV) (4-8) as well as human cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (9, 10). Several of these standards, like those for HCV, HBV and HIV-1, have been essential for introducing regulatory requirements for testing of blood and plasma donations as well as being used by clinical microbiology laboratories for determination of viral loads. In the field of transplantation, ISs have been prepared for CMV, EBV, BK virus (BKV), JC virus (JCV) as well as human herpesvirus type 6b (HHV6b) (9-13). Other ISs established include ones for the parasites *Plasmodium falciparum* and *Toxoplasma gondii* (14, 15) as well as a standard for *Mycoplasma* species (16). More recently, emerging diseases have been addressed with the establishment of ISs for Zika virus (ZIKV) and chikungunya virus (CHIKV) (17, 18). Slightly different types of WHO standards, termed reference reagents have been prepared for Ebola virus (19) and the four different dengue virus serotypes (20). Although initially developed for vaccine studies, ISs have been prepared for human papillomavirus type 16 and 18 (21), in this case, based on plasmids representing the viral genomes due to lack of native or cultured source materials. Current WHO ISs and reference reagents for NAT are shown in Table 1.

The SoGAT group has met at least annually since it was established, collectively identifying priority pathogens where there is a need for NAT-standardization and coordinating international studies to develop and evaluate these materials. The need for specific standards is determined through discussions with the scientific and medical community worldwide through the SoGAT forum, through WHO programs in disease areas such as malaria and tuberculosis, with input from manufacturers of *in vitro* diagnostic devices (IVDs) and by the

three official WHO collaborating centers in the fields of blood and IVDs: NIBSC, the Paul-Ehrlich-Institut (PEI, Germany) and the U.S. Food and Drug Administration, Center for Biologics Evaluation and Research (FDA/ CBER, USA).The SoGAT meetings allow for the discussion of results from international collaborative studies prior to submission and review by the WHO Expert Committee on Biological Standardization (ECBS). The ECBS plays a formal role in the establishment of ISs and related reference materials, and committee members are scientific experts from national control agencies, research institutes, academia, public health bodies and the pharmaceutical industry. All new proposed international standardization projects are subject to review by the ECBS before endorsement. Occasionally, special topics have been discussed at extraordinary SoGAT meetings; examples include addressing the problems with detection of different genotypes of B19V and how to improve standardization (22).

TYPES OF WHO REFERENCE MATERIALS

International Standards (ISs) and their role

ISs are measurement standards and are assigned an internationally agreed unitage in IU (23). The potencies of ISs are determined by consensus means through international collaborative studies, using a range of methods typically in routine use by participating laboratories. In the case of NAT assays, potencies are determined by a combination of end-point dilution analysis for qualitative assays and, for example, by “copy numbers” or “genome equivalents” for quantitative assays. Although the IU is arbitrary in theory, in practice, it corresponds to the mean overall potency (“NAT-detectable units”) reported by participating laboratories. Adoption of the IU also avoids the issue of copy number, the definition of which is assay-dependent and which also implies, misleadingly, that material is traceable to an SI unit. Repeatedly, during studies to evaluate new ISs, quantitative reporting of concentrations of samples in copy numbers typically varies over several orders of magnitude. This demonstrates

that copy number is not a robust measure that can be compared readily between laboratories; the use of the IU allows better comparison of results.

WHO ISs are considered as the highest order, international conventional calibrators in accordance with ISO 17511:2003 (*In vitro* diagnostic medical devices - Measurement of quantities in biological samples - Metrological traceability of values assigned to calibrators and control materials) (24). The principal use of ISs is in the calibration of secondary standards (Figure 1), traceable in IU and for evaluation of critical assay parameters such as analytical sensitivities and quantification range, including upper and lower limits of quantification. The preparation and calibration of secondary standards is described in detail elsewhere (25). Uncertainty values are not assigned to WHO ISs, since the IU is an arbitrary unit and variance is associated with that of the vial content.

In Europe, the new Regulation on *in vitro* diagnostic medical devices (CE-IVDs) stipulates the design requirements for calibration of assays using "reference materials of a higher metrological order" (26). Furthermore, the Regulation requires metrological traceability of values assigned to calibrators and control materials using "reference materials…of higher order" which should be communicated to the user. In addition, the "Common Technical Specifications" state that WHO ISs should be included in the performance evaluation and the reporting of test results in IU for "high risk" IVDs (e.g. for quantitation of HIV-1, HBV, or HCV) (27). Furthermore, regulatory requirements for testing of biologics may define minimal sensitivity for suitable assays based on WHO ISs. Examples are national requirements for blood screening markers (e.g. HIV-1 RNA, HCV RNA in Germany) or European regulation of plasma derivatives (e.g. HCV RNA in manufacturing plasma pools).

Representatives of the US FDA/CBER participate on a regular basis in the international standardization efforts undertaken by WHO. In contrast to the EU, there is no legal

requirement in the US to use WHO ISs for assay calibration; however, panel members used by FDA/CBER for lot release of NAT tests have been calibrated against WHO ISs (28, 29). When an IS is established for the first time, it is designated the 1st IS, upon its replacement it is termed the 2nd IS, the 3rd IS and so on and with each subsequent standard replacing its predecessor as the highest order reference standard. Replacement of ISs is discussed in more detail below.

Reference reagents and international reference panels

In addition to WHO ISs, there are other types of standards established by the WHO ECBS, these include Reference Reagents (RRs) as well as International Reference Panels (IRPs). Both RRs and IRPs are prepared and evaluated using principles similar to WHO ISs.

The IRPs consist of different genotypes or important strains of pathogens with diverse global distribution; examples of such panels include HIV, HBV, B19V and HEV (Table 2) (30-35). The role of IRPs is to help ensure consistent detection of pathogen variants, particularly when being used for assay validation purposes. They have been important tools for improvement in assay performance where detection of specific variants has been sub-optimal. Usually, no unitage is assigned to members of IRPs. However, the data on assay performance are included in the collaborative study reports published on the WHO website, providing a range of potencies reported for individual panel members.

In the case of RRs, these are usually interim standards with a unitage defined in units rather than IU. Upon further characterization, RRs may be established as ISs and the unitage defined in IU. Examples of RRs include NAT standards for Ebola virus, established in response to the Ebola crisis in 2014, and based upon recombinant lentivirus vectors to avoid biosafety issues (19). More recently, four RRs have been established for dengue types 1-4; because of the

genetic differences between the types it was not possible to select a single strain as an IS, consequently each type has a separate unitage (20).

PREPARATION AND ESTABLISHMENT OF WHO REFERENCE MATERIALS

Characterization and preparation of candidate standards

The processes involved from the identification of the scientific need to develop a standard through establishment and ultimately its replacement are shown in Figure 2. The procedure to establish WHO standards is extremely rigorous (23) and undertaken by one of the three WHO Collaborating Centers on behalf of the WHO.

The development of a new standard starts with the identification and preparation of a suitable stock material which may either be viremic plasma – for example for HCV, HBV and HEV or parasitemic whole blood (*Plasmodium falciparum*) (14), or pathogens propagated in culture. More rarely, animals have been used as alternative starting materials where sources of native materials are unavailable or not of insufficiently high titer, and example of this is the propagation of *Toxoplasma gondii* tachyzoites in mice (15). HPV ISs have been based on the preparation of plasmid DNAs diluted in human genomic DNA (21). An estimate is made of the concentration of the stock material and identity testing is performed e.g. by sequence analysis, and where material has been obtained from blood or plasma, donations are screened to ensure the absence of other blood-borne pathogens other than the target in question. Strains are selected to reflect those with widespread distribution and global importance whenever possible. Occasionally, materials may be inactivated depending on feasibility combined with biosafety concerns; such procedures should be validated, however, this may not be possible for some pathogens where suitable cell culture systems are not available. To facilitate distribution worldwide, WHO standards are usually lyophilized. Therefore, formulation is an important factor to consider and this is fairly straightforward where viremic plasma is used

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and the standards will be further diluted in this matrix when used in the recipient laboratories. However, where testing of certain pathogens can be performed on different types of matrices, e.g. whole blood, urine, cerebrospinal fluid (CSF) as well as plasma, cultured viral and microbial strains have been formulated in solutions containing excipients (buffers, sugars, stabilizers etc.) that allow further dilution of the standard into the appropriate type of matrix. The final formulation should not cause any interference with the NAT assays, e.g. decrease in extraction efficiency or inhibition of amplification.

When the bulk standard preparation is dispensed into either vials or ampoules, the coefficient of variation of the filled volume is determined. Several thousand vials/ampoules are usually prepared. After lyophilization, the ampoules or vials are back-filled with nitrogen and the homogeneity of the lyophilized material is determined, sampling across the batch. Testing is performed for residual moisture and oxygen which may impact product stability and accelerated (at higher temperatures) and real-time stability is determined to ascertain that the reference material can be shipped at ambient temperatures worldwide, without loss of potency under normal storage temperatures (typically -20°C) over the life of the IS.

Commutability

Commutability is a property of a reference material demonstrated by the closeness of agreement between the results obtained for the reference material and the results obtained for clinical specimens, when comparatively tested in different assays (36, 37). In other words, in order to be suitable as an assay calibrator, the reference material should not behave differently compared to clinical specimens. Commutability is demonstrated by testing the different materials (reference material, clinical specimens) in multiple assays. ISs are designed to reflect as closely as possible the specimens tested in routine diagnosis or blood screening. For example, human plasma or sera are very common types of sample matrices tested in blood screening and clinical laboratories and several ISs are derived from viremic donations or

contain culture-derived virus diluted in plasma. In addition, the strain of pathogen (i.e. the analyte) used for the IS is usually selected to represent the most commonly circulating variant. Commutability is an important precondition for the ability of the calibrant to harmonize different assays, and is addressed by inclusion of clinical specimens, as far as possible, in the international collaborative study. The impact of different extraction systems (reagents, equipment) on the extraction efficiencies for different matrices is another factor to be addressed in commutability studies. In the case of CMV, non-commutability of the IS has been demonstrated for some assays (38). Commutability, in the case of CMV, is particularly complex and affected by features such as the physical form of viral DNA in the IS (virion-associated DNA) compared to that found in transplant patients which is highly fragmented (39, 40). Furthermore, during amplification/detection reactions, amplicon length impacts viral load determinations (40). With the development of additional IS for clinical pathogens the challenge of commutability becomes even more complex with quantitative values reported for multiple types of sample matrices, including urine, CSF and stool. In the case of CSF, it is a matrix with a low protein content which is difficult to obtain in large volumes, and is not easy to evaluate in collaborative studies or in formal commutability investigations. Stool is another challenging sample type where the matrix contains inhibitors and the sample extraction is not well standardized.

International collaborative studies

Candidate ISs, RRs and IPRS are evaluated in international collaborative studies. Participants volunteering to take part in these studies include blood centers, reference laboratories, clinical microbiology laboratories, manufacturers of diagnostics kits and medicinal products as well as regulatory organizations. Typically 15 to 25 laboratories will be involved in such a study. The assays included in the studies are ones used throughout the world, and include commercially available tests as well as LDTs. The studies investigate potency of the candidate materials, clinical comparator samples as well as related reference materials and

calibrators; potencies are determined using qualitative or quantitative assays as described above. One of the major aims of each study is to provide a basis for assignment of unitage to the standard; the unitage assignment is usually based on the combined mean potency for all the assays included in the study. Expressing results of the study samples against the candidate IS can greatly reduce variation in the measured potencies reported by participants, and the harmonization effect (see below) is an important factor reviewed by the ECBS to demonstrate the utility of a new IS. The studies themselves allow a head-to-head comparison of assays used throughout the world and provide information on sensitivity (based on end-point analysis of qualitative assays) as well as variability in quantification.

Statistical analysis of the study data forms the basis for the final report which includes a proposal for the unitage for the IS. Participants are requested to comment on the report and asked if they agree with the proposed unitage. The final report is made available on the WHO website for public review ahead of the annual meeting of the ECBS. In the case of IRPs, no unitage is assigned to the panel members; however, details may be included in the report with the range of potencies observed.

Subsequent to the establishment of a standard or panel, the custodian laboratory has a responsibility for the storage of each batch under controlled conditions, monitoring of stability and coordinating distribution worldwide.

REPLACEMENT OF WHO ISs

Although several thousand vials are prepared for each standard, when they are nearing exhaustion, it is essential to replace the previous preparation. Replacement projects are prioritized by the WHO. An important aspect of replacement of one standard with the next is maintaining the continuity of the IU in order to ensure that tests can be compared over time. Details of the NAT standards which have been replaced are shown in supplementary Table S1. Since it was established in 1997 (1), the HCV IS has been replaced four times (41-44).

Replacement ISs have been prepared for HBV (45-47), HAV (48, 49), HIV-1 (50-52) and B19V (53, 54). In each case, replacement preparations have been evaluated in parallel with the previous IS, using either qualitative end-point assays or quantitative assays (within the linear range) and covering appropriate dilutions. With each subsequent IS, the possibility exists for drift in the IU; this may be exacerbated by issues with assay features included in collaborative studies, such as primer/probe mismatches affecting quantification, and emphasizes the need for good characterization of starting materials. An example is the study to establish the 3rd IS for B19V (54) where the new B19V viremic plasma donation used for the 3rd IS was under-quantified by the COBAS TaqScreen DPX test, probably due to a mismatch between the primers/probe and the sequence of the ISs (55), impacting the assigned unitage.

ASSAY HARMONIZATION USING WHO ISs

Relative potencies

During the establishment of WHO ISs, one of the criteria for acceptance of a new standard is the demonstration that when results of testing are expressed relative to the candidate IS, an improvement is seen in the agreement observed between assays and laboratories.

An example of this is shown in Figure S1. A HEV sample, included in the collaborative study to establish the HEV IS, was evaluated using a mixture of qualitative and quantitative NAT assays – the reported potencies are shown in the upper panel showing a wide variation in titres over several orders of magnitude. By expressing these potencies against the WHO IS (PEI code number 6329/10) the agreement between laboratories is markedly improved with variation being reduced to ~ 1 $\log_{10}$ and a typical reduction in the associated standard deviation (SD).

E*xternal Quality Assessment Programs*

External quality assessment (EQA)/proficiency testing (PT) programs can be very helpful in generating data on the implementation of WHO ISs by participating laboratories in a large number of countries. In some cases, WHO ISs have been included directly in EQA studies. For example, the 1st IS for ZIKV was made available by the WHO in July 2016 prior to formal establishment by the ECBS and was introduced as a consequence of the Public Health Emergency of International Concern (56). The 1st ZIKV IS has been included in all the ZIKV EQA/PT programs provided by Quality Control for Molecular Diagnostics (QCMD) since 2016 (57).

Data analysis from QCMD EQA/PT schemes demonstrate that where an IS has been established for a specific target pathogen the observed variation (SD) based on the geometric mean of the $\log_{10}$ viral load results, are noticeably smaller (Table S2). This observation is based on results reported in IU/mL on duplicate panel members. In contrast, for pathogen targets where an IS has only recently been established or where there is no IS and reporting of results is often in different types of unit, the SDs are much greater (Table S2). In addition, where there is a known clinical need for pathogen quantitation then the IS and IU/mL are more readily accepted.

In the case of CMV, for example, in early EQA/PT studies done prior to 2004, the majority of assays performed by laboratories participating in the CMV EQA program were qualitative (Figure S2). For quantitative assays performed prior to the establishment of the 1st CMV IS in 2010 (9), laboratories reported results in either copies/mL or other units of measurement such as genome equivalents/mL as observed through the data reported in international EQA/PT schemes. Over the last 8 years the number of laboratories reporting in IU/ml has increased significant from 0 to 50% of the datasets returned within the annual international EQA/PT schemes run by QCMD (Figure S3). For CMV viral load testing, the increase in reporting in IU correlates with an increase in the use of commercial assays used by participants in the QCMD studies (Figure S4). In a recently published EQA study, evaluating results reported in

IU/mL, the variation between results was lower when compared to those reported in copies/mL demonstrating the use of the CMV WHO IS improves the reproducibility and comparability of CMV viral load results across laboratories (58). Consequently, the recently revised International guidelines on the management of CMV in solid organ transplantation recommend that all results should be reported as IU/mL (59). More significant improvements in results have been reported for EBV when the IS has been used (60).

PRE-QUALIFICATION OF *IN VITRO* DIAGNOSTIC DEVICES

International reference preparations play an important role in the WHO prequalification program for IVDs. In this program, IVDs targeting low- and middle-income countries (LMIC) are independently assessed by WHO since LMIC themselves rarely have the regulatory capacity to assess the quality and suitability of IVDs offered to the national market. In WHO prequalification studies, ISs may be used for comparative evaluation of essential assay features such as sensitivity, limit of detection or range of quantitation. Furthermore, IRPs covering different variants (e.g. genotypes, recombinants) are important for the detection of strains more prevalent in certain regions. The outcome of performance evaluation studies initiated on behalf of the WHO prequalification program for IVDs is published together with a list of IVDs deemed suitable by WHO for the intended purpose.

STRATEGIC ADVISORY GROUP OF EXPERTS ON *IN VITRO* DIAGNOSTICS (SAGE IVD)

In 2017, the WHO established the Strategic Advisory Group of Experts on *In Vitro* Diagnostics (SAGE IVD). SAGE IVD recently published the first model list of essential diagnostics, including several NAT assays for markers including HBV, HCV, HIV, *Mycobacterium tuberculosis* and HPV (61). The elaboration of the list is aimed to improve

access to IVDs which are estimated essential in a given health system. The ultimate goal is strengthening of health systems and the availability of universal health coverage. This is akin to the WHO essential medicines list which includes those medicines which are deemed indispensable in a health care system.

STANDARDS CURRENTLY UNDER DEVELOPMENT

Standards currently under development are shown in the supplementary Table S3 and include viral and parasitic markers as well as a standard for *M. tuberculosis* reflecting the global burden of disease and the increasing use of molecular testing for this pathogen.

CONCLUSIONS

Significant progress has been made in NAT standardization over the past two decades in the context of screening for blood-borne markers as well as in clinical diagnostic laboratories. The development of WHO standards and other reference reagents (ISs, RRs and IRPs) has helped in these efforts, also enabling the introduction of regulations for the detection of blood-borne pathogens in the fields of transfusion and blood product safety for markers such as HCV, HBV, HIV, HAV, B19V and more recently HEV by setting thresholds and control concentrations, defined in IUs. For clinical laboratories, for diagnosis and treatment monitoring, HCV, HBV and HIV-1 standards have been important for viral load determinations; in relation to transplantation standards established for CMV, EBV, HEV, BKV, JCV and HHV-6b are used for expression of viral loads in IU. The use of the IU improves agreement and allows comparability of data between laboratories and allows the introduction of regulations in blood screening using NAT and informs clinicians in patient testing and monitoring of therapeutic interventions. International clinical guidelines e.g. for

CMV and HEV in the transplant setting, reporting in IU is encouraged further supporting accuracy in viral load reporting and harmonization efforts (59, 62). These efforts are underpinned by the secondary standards and controls traceable in IU as well as calibrated assays.

Because of their biological nature, WHO standards control for the entire NAT process – including nucleic acid extraction. Organizations such as the National Institute of Standards Technology in the US, take a different approach and produce "standard reference materials" (SRMs) for a small number of viral markers including a bacterial artificial chromosome (BAC), containing the genome of the CMV Towne strain and a linearized plasmid DNA control for BK virus. These SRMs are added directly to the amplification/detection reaction without undergoing prior extraction and are intended to be used for the calibration of controls and standards. Some organizations provide *in vitro* transcribed RNAs (IVTs), and like the NIST materials these materials do no control for the extraction part of the NAT assay. In a study organized by kit manufacturers, a partial HCV IVT RNA was evaluated in a study comparing amplification methods; however it was not found to perform better than the biological standard (63). During the study to establish the 1st WHO IS for CMV, the candidate standard, based on a clinical strain (Merlin) propagated in cell culture, was evaluated in parallel with BAC containing the entire Merlin genome. Participants added the BAC directly to the amplification reactions. Expression of potencies of other cultured virus preparations against the candidate IS showed marked reduction in variation between laboratories, however, when the results were expressed relative to the BAC no improvement was observed compared to the absolute mean estimates (9). In the study to establish the 1st WHO IS for ZIKV, expression of clinical samples and biological reference materials saw an improvement in agreement of results between laboratories. In the study, two related IVTs were included – one containing several assay target sequences in a single transcript and the second preparation a mixture of the respective individual IVT RNAs. Expressions of the one IVT preparation

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against the other resulted in harmonization, however, expression of clinical samples or biological reference materials against the IVTs failed to produce any improvement (17). These studies demonstrate the importance of controlling the extraction step in the NAT procedure and emphasizes the advantage of the approach taken by the WHO compared with (bio)-synthetic types of reference material. However, the latter may be easier to replace compared to sourcing, for example, new viremic donations in the case of some of the WHO ISs.

Sequence data is available for most WHO ISs, RRs and IRPs (Table 1, supplementary information and Tables S4-S7), sometimes indicating sequence heterogeneities when compared to clinical isolates, e.g. sequence deletions or sequence duplications in culture based materials. Using next generation sequencing data, even subpopulations of sequence variants are being detected, as was reported recently for the ISs BK and JC polyoma viruses (64, 65). Passage of the strains in cell culture resulted in heterogeneous DNA populations, the reason for which is not understood and which could affect some specialized assays (64, 65), although both preparations were shown to successfully harmonize assay performance in the collaborative studies (11, 12) and in independent studies (66). These observations demonstrate the importance in thorough characterization of the starting materials used for standard preparation. Methods such as digital PCR are useful in the characterization process in understanding the relationship between IU and copy number ratios for specific methods as well as for estimating potency during development of new ISs or when no standard exists. In the case of the 1st WHO IS for HAV, the IU:copy number ratio was determined to be 1:14 using digital PCR (S. Baylis unpublished data) and the low IU value was a consequence of low sensitivity of assays used by participants in the original collaborative study (5).

With the absence of reference methods to define nucleic acid content of microbial pathogens in complex biological matrices, this emphasizes the validity of WHO approach in the

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development of reference standards and harmonizing NAT assays. However, the challenge for the development of such standard remains meeting the clinical need in a timely manner whilst maintaining rigorous procedures in the establishment process. Adequate commutability of ISs is essential particularly in the clinical setting and will affect treatment of patients and hinder the introduction of clinical practice guidelines. Inclusion of sufficient clinical materials in studies to evaluate commutability remains a problem in terms of volume, transfer agreements and the support of the wider scientific community in these efforts is essential to fully realize the potential of the WHO standardization efforts.

ACKNOWLEDGMENTS

We gratefully acknowledge the essential contribution of all collaborative study participants over the years.

FOOTNOTES

Details of the reference preparations are available on the WHO website as well as on the respective collaborating centers websites.

435 **FIGURE LEGENDS**

436 Figure 1 Hierarchy of standards

437 The relationship between ISs and secondary and tertiary standards is shown together with
438 their uses.

439 Figure 2 Process for the development of WHO ISs, RRs and IRPs

440 The procedure is shown from the identification of a scientific need to develop a standard to its
441 establishment and ultimately its replacement. cIS – candidate International Standard.

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Table 1 Current viral and microbial WHO International Standards and Reference Reagents for NAT

International Standards and Reference Reagents for NAT				
Preparation (unitage)	**Standard (code number)**	**Material (accession no.)***	**Year of establishment**	**Reference**
BK virus DNA (10,000,000 IU/vial)	1st International Standard (14/212)	Cultured BK virus, diluted in buffer/human serum albumin/trehalose	2015	11
Chikungunya virus RNA (1,250,000 IU/vial)	1st International Standard (11785/16)	Cultured and heat inactivated R91064 strain diluted in human plasma (KJ941050).	2017	18
Dengue virus RNA (13,500 units/vial)	1st Reference Reagent	Cultured and heat inactivated Hawaii strain diluted in human plasma (KM204119).	2016	20
Dengue virus RNA (69,200 units/vial)	1st Reference Reagent	Cultured and heat inactivated New Guinea C strain diluted in human plasma (KM204118).	2016	20
Dengue virus RNA (23,400 units/vial)	1st Reference Reagent	Cultured and heat inactivated H87 strain diluted in human plasma (KU050695).	2016	20
Dengue virus RNA (33,900 units/vial)	1st Reference Reagent	Cultured and heat inactivated H241 strain diluted in human plasma (KR011349).	2016	20
Ebola virus NP-VP35-GP (32,000,000 units/vial)	1st Reference Reagent (15/222)	Lentiviral vector encoding Ebola genes np-vp35-gp in buffer/human serum albumin/trehalose (KT186367).	2015	19
Ebola virus VP40-L (50,000,000 units/vial)	1st Reference Reagent (15/224)	Lentiviral vector encoding Ebola vp40-L genes in buffer/human serum albumin/trehalose (KT186368).	2015	19
Epstein Barr virus DNA (5,000,000 IU/vial)	1st International Standard (09/260)	Cultured EBV B95-8 strain, diluted in buffer/human serum albumin/trehalose (V01555).	2011	10

International Standards and Reference Reagents for NAT				
Preparation (unitage)	**Standard (code number)**	**Material (accession no.)***	**Year of establishment**	**Reference**
Hepatitis A virus RNA (15,451 IU/vial)	3rd International Standard (15/276)	Viremic human plasma (KY003229).	2017	49
Human cytomegalovirus DNA (5,000,000 IU/vial)	1st International Standard (09/162)	Cultured Merlin strain, diluted in buffer/human serum albumin/trehalose (AY446894).	2010	9
Hepatitis B virus DNA (477,500 IU/vial)	4th International Standard (10/266)	Viremic human plasma representing HBV genotype A2, HBsAg subtype adw2 (KY003230).	2016	47
Hepatitis C virus RNA (100,000 IU/vial)	5th International Standard (14/150)	Viremic human plasma representing HCV genotype 1	2015	44
Hepatitis D virus RNA (287,500 IU/ml)	1st International Standard (7657/12)	Viremic human plasma (HQ005369).	2013	8
Hepatitis E virus RNA (125,000 IU/vial)	1st International Standard (10/6329)	Viremic human plasma representing HEV genotype 3a (AB630970).	2011	7
HIV-1 RNA (125,893 IU/vial)	4th International Standard (16/194)	Cultured and heat inactivated subtype B isolate diluted in human plasma (KJ019215).	2017	52
HIV-2 RNA (1,000 IU/vial)	1st International Standard (08/150)	Cultured and heat inactivated CAM2 strain diluted in human plasma (KU179861).	2009	6
Human Herpes Virus 6B (56,234,132 IU/vial)	1st International Standard (15/266)	Cultured HHV-6B strain Z-29, diluted in buffer/human serum albumin/trehalose (AF157706).	2017	13
Human papilloma virus type 16 DNA (5,000,000	1st International Standard (06/202)	HPV type 16 plasmid DNA diluted in buffer/trehalose (K02718).	2008	21

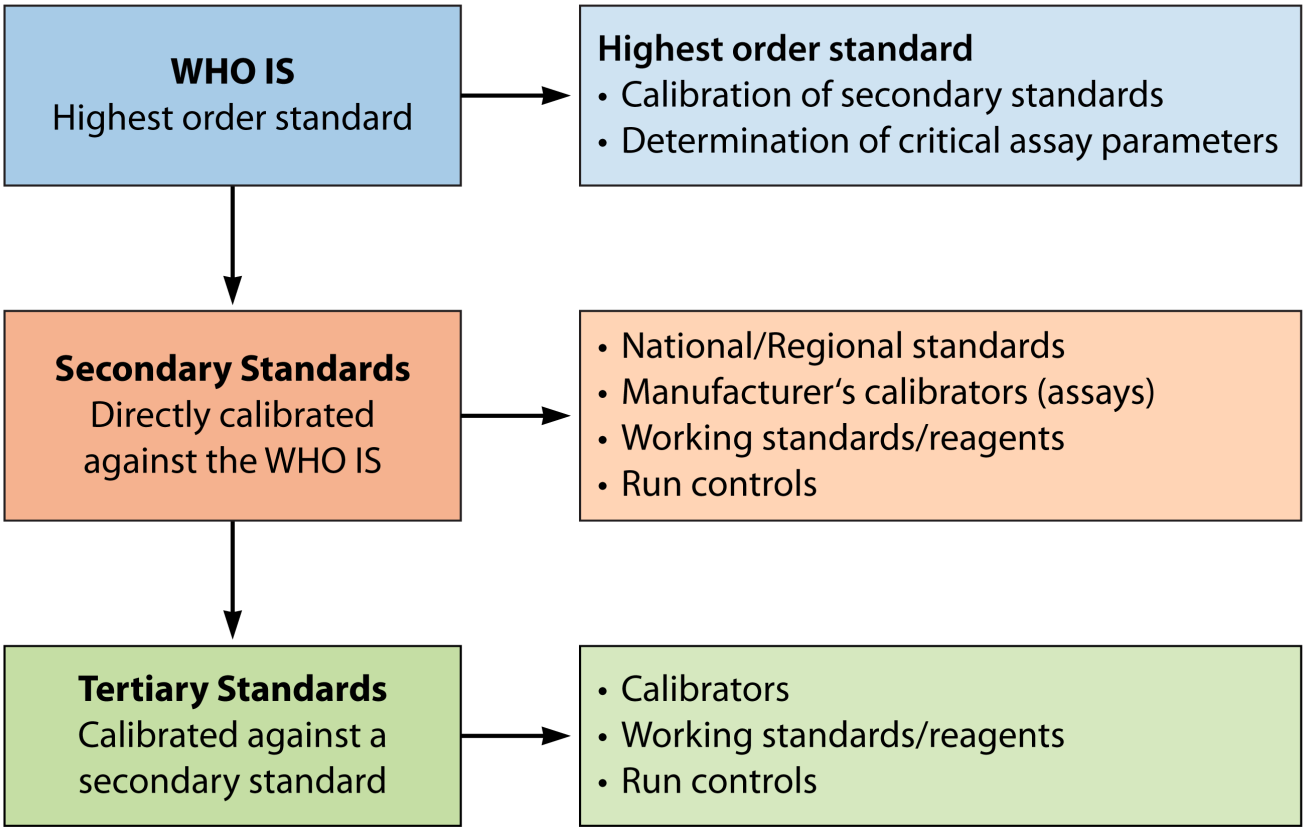
International Standards and Reference Reagents for NAT				
Preparation (unitage)	**Standard (code number)**	**Material (accession no.)***	**Year of establishment**	**Reference**
IU/vial)				
Human papilloma virus type 18 DNA (5,000,000 IU/vial)	1st International Standard (06/206)	HPV type 18 plasmid DNA diluted in buffer/trehalose (X05015).	2008	21
JC virus DNA (10,000,000 IU/vial)	1st International Standard (14/114)	Cultured JC virus, diluted in buffer/ human serum albumin/trehalose	2015	12
Mycoplasma DNA (100,000 IU/vial)	1st International Standard (8293/13)	Cultured *Mycoplasma fermentans*, in Mycosafe Friis medium	2013	16
Parvovirus B19 DNA (705,000 IU/vial)	3rd International Standard (12/208)	Viremic human plasma representing B19 genotype 1	2013	54
Plasmodium falciparum DNA (500,000,000 IU/vial)	1st International Standard (04/176)	Parasitemic human blood	2006	14
Toxoplasma gondii (500,000 IU/vial)	1st International Standard (10/242)	*T. gondii* tachyzoites obtained from infected mice, diluted in buffer/trehalose	2014	15
Zika virus RNA (25,000,000 IU/vial)	1st International Standard (11468/16)	Cultured and heat inactivated PF13/251013-18 strain diluted in stabilizer (KX369547).	2016	17

*Sequences are unavailable for some ISs

Table 2 Current International Reference Panels for NAT (viral markers)

International Reference Panels for NAT				
Panels (No. of members)	**Standard (code number)**	**Material**	**Year of establishment**	**Reference**
Hepatitis B Virus genotypes (15)	1st International Reference Panel (5086/08)	Viremic plasma diluted in pooled human plasma; HBV genotypes A-G	2009	33
Hepatitis E virus genotypes (11)	1st International Reference Panel (8578/13)	Viremic plasma donations and stool samples diluted in pooled human plasma; HEV genotypes 1a, 1e, 2a, 3b, 3c, 3e, 3f/l, 3 ra, 4c, 4g	2015	34
HIV-1 subtypes (10)	1st International Reference Panel (01/466)	Cultured HIV-1 subtypes A, B, C, D, AE, F, G, AG-GH, N and O diluted in human plasma	2003; replaced in 2012 by 12/224	30
HIV-1 subtypes (10)	2nd International Reference Panel (12/224)	Cultured and heat inactivated HIV-1 subtypes A, B, C, D, AE, F, G, AG-GH, N and O diluted in human plasma	2012	31
HIV-1 circulating recombinant forms (10)	1st International Reference Panel (13/214)	Cultured and heat inactivated HIV-1 CRFs and subtype variants diluted in human pooled plasma	2013	32
Parvovirus B19 genotypes (4)	1st International Reference Panel (09/110; CBER Parvovirus B19 Genotype Panel 1)	Viremic plasma donations diluted in pooled human plasma; B19V genotypes 1a1, 2, 3a and negative plasma control	2009	34

*Sequence details for IRP members are available in supplementary information (text and Tables S4-S7).



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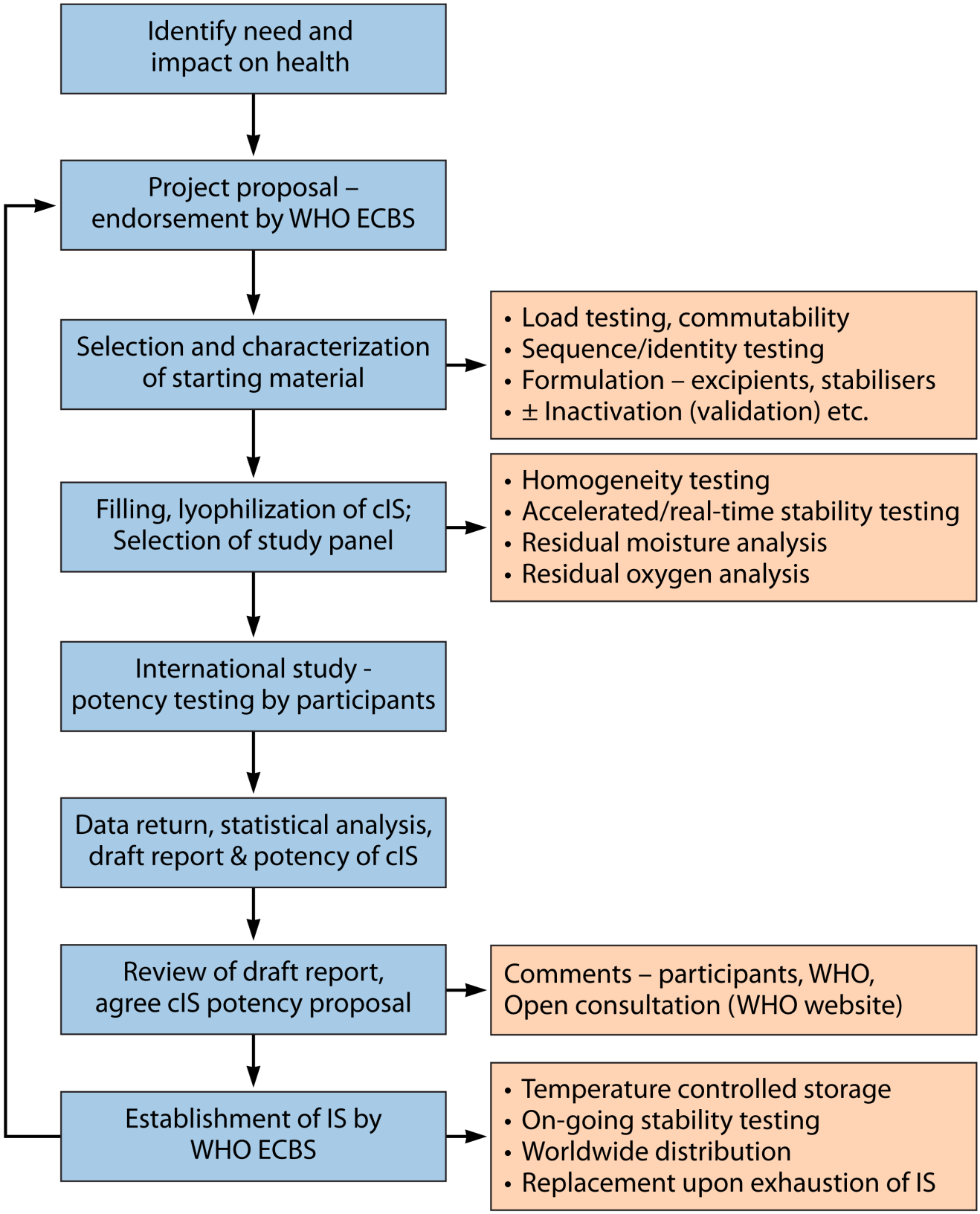

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