

# Review

## International Standards and Reference Materials for Quantitative Molecular Infectious Disease Testing

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**The utility of quantitative molecular diagnostics for patient management depends on the ability to relate patient results to prior results or to absolute values in clinical practice guidelines. To do this, those results need to be comparable across time and methods, either by producing the same value across methods and test versions or by using reliable and stable conversions. Universally available standards and reference materials specific to quantitative molecular technologies are critical to this process but are few in number. This review describes recent history in the establishment of international standards for nucleic acid test development, organizations involved in current efforts, and future issues and initiatives. (J Mol Diagn 2010, 12:133–143; DOI: 10.2353/jmoldx.2010.090067)**

In the 1990s, it was demonstrated that HIV viral load testing could predict and monitor the course of HIV disease.<sup>1,2</sup> Similar studies followed for other viruses, and now quantitative molecular tests are integral to the prognosis and monitoring of several infectious diseases.<sup>3–5</sup> Early assays, though, lacked adequate reproducibility and comparability, as illustrated in proficiency testing reports. This inadequacy was attributed to the abundant use of laboratory-developed protocols, inconsistent training, limited usage of standardized industry-produced assays, and the absence of adequate universal standards for test development.<sup>6–8</sup>

To mitigate persisting differences, current clinical practice recommendations have been developed whereby serial specimens from patients are optimally tested with the

same assay each time they are submitted.<sup>9</sup> However, the need for all tests to report consistently similar results is still critical and is driven by the increasing length of time that patients are monitored, the decreasing ability of practitioners to demand the use of specific laboratories and tests, diluted knowledge of these practice standards among the increasing number of nonspecialist treatment providers, and the fact that some medical decisions are based on absolute values instead of relative changes. The requirement for translatable quantification among different molecular tests relies on universally available, robust standard reference materials for test developers, manufacturers, and proficiency test providers.

Although molecular diagnostics have contributed to patient management for more than a decade, the establishment of standards and reference materials to support the field have lagged. The current World Health Organization International Standards address some of the needs, but they cover only six viruses and one bacterial target (National Institute for Biological Standards and Control, <http://www.nibsc.ac.uk>, last accessed August 20, 2009). These materials and the collective efforts to establish them have played a critical role, particularly in evaluating the sensitivity of molecular screening assays and in facilitating the relative standardization of quantitative assays. However, because of their original intended use as sensitivity standards for plasma product manufacturers' qualitative assays, they lack some of the characteristics desired for the standardization of quantitative diagnostic assays. Another possible resource, the Joint Committee on Traceability in Laboratory Medicine (JCTLM), which was established to "promote and give guidance on internationally recognized and accepted equivalence of measurements in laboratory medicine and traceability to appropriate measurement standards" maintains a

Accepted for publication September 4, 2009.

Certain commercial equipment and materials are identified to specify the experimental procedure. This does not imply endorsement by the National Institute of Standards and Technology nor does it imply that the material or equipment is the best available for the purpose.

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database of certified reference materials and reference methods (Joint Committee on Traceability in Laboratory Medicine, <http://www.bipm.org/en/committees/jc/jctlm>, last accessed August 20, 2009). However, there are currently no standards for infectious disease quantitative molecular diagnostics either at the level 1 category of high-order reference materials (the level of purified chemicals) or in their level 2 category, which is designed to catalog biological products and consensus calibrated materials. Laboratory test developers and *in vitro* diagnostic (IVD) test manufacturers are left with very few options.

In this review we discuss the establishment of the current World Health Organization International Standards, the requirements needed specifically for quantitative molecular diagnostics, and current global efforts to address these needs. Through scientific, political, and pragmatic perspective guide opinions regarding these developments, we attempt to present them factually and to provide the clinical community with a foundation for future informed discussions.

### **Characterization, Establishment, and Replenishment of the World Health Organization International Standards**

The World Health Organization International Standards are prepared, characterized, and established according to "The World Health Organization Guidelines for the Preparation and Establishment of Reference Standards for Biological Substances."<sup>10</sup> The first version of this document was written in 1978. It was revised in 1986, in 1990, and most recently in 2004 in a series of meetings with representatives from country and regional health departments, vaccine manufacturers, standards organizations, and diagnostic test manufacturers. It describes the general principles for the establishment of all World Health Organization biological reference materials, with a broad scope of intended uses: primarily vaccine preparations and immunological and biological assays. The quantification of viral targets in molecular testing is recognized in the most recent revision.

The most commonly used global standards for the calibration and characterization of quantitative molecular viral assays are the World Health Organization International Standards for hepatitis C virus (HCV), HIV, and hepatitis B virus. The World Health Organization International Standard (IS) for HCV was the first of the series and was established in 1997. It was a preparation of an HCV *genotype 1a* high-titered plasma unit diluted into cryosupernatant and lyophilized. The preparation was tested with two other candidate materials in a global collaborative study in 22 laboratories during 1996. The study was directed by and the data were analyzed by the World Health Organization collaborating center laboratory: the National Institute for Biological Standards and Controls (NIBSC) in South Mimms, UK. The methods examined included a variety of commercial and laboratory developed assays using single, nested, or heminested primers. Most of the data were generated by testing endpoint

dilutions of the candidate standards using qualitative traditional (non-real-time) amplification assays, although quantitative data submitted by participants were included in the analysis. The first World Health Organization HCV IS (96/790) was assigned the value of  $10^5$  IU/ml ( $5 \log_{10}$  IU/ml) lyophilized in 0.5-ml ampoules.<sup>11</sup> The international unit is considered absolute, and, consistent with World Health Organization policies, there are no units of uncertainty associated with this or subsequent replacements. The IU for each biological preparation "has no existence other than in relation to the preparation that defines it."<sup>10</sup>

Since 1997, the first HCV IS has been replaced by two subsequent standards: the second World Health Organization HCV IS (96/798) in 2003, which was a second lyophilization preparation of the original material<sup>12</sup> and most recently a new material prepared and tested in 2007.<sup>13</sup> The second HCV IS (96/798) was originally tested in the global collaborative study of 1996. Because it was the same material as the first IS and was determined in 1996 not to be statistically different from the first, there was no global collaborative study when it assumed its place as the second IS 6 years later. Stability and comparative studies were performed in three selected laboratories before its use. The second World Health Organization HCV IS (96/798) was established with the same value as the first (96/790):  $10^5$  IU/ml ( $5 \log_{10}$  IU/ml).

For the recent replacement of the second by the third HCV IS, new materials were commissioned, and a full global replacement study directed by NIBSC was performed. The candidates were two lyophilized and one frozen preparation of a genotype 1a, anti-HCV-negative, HCV RNA-positive plasma unit diluted into human plasma. The candidate materials were tested with the second HCV IS (96/798) by 33 participating laboratories in 14 countries. This time most assays were quantitative, although data testing the limiting dilutions of the materials with qualitative molecular assays were also included. In addition, real-time amplification assays not present in the first study more than 10 years earlier had become common and were reflected in the submission of data. The mean quantification of the second HCV IS (96/798) was  $5.10 \log_{10}$  IU/ml with a 95% confidence interval of 5.02 to  $5.17 \log_{10}$  IU/ml, which is statistically different from the previously assigned  $5.0 \log_{10}$  IU/ml. The mean values of the two lyophilized candidates were  $5.19 \log_{10}$  IU/ml (sample 2) and  $5.47 \log_{10}$  IU/ml (sample 3). Sample 2 (NIBSC code 06/100) was recommended as the third HCV IS to the Expert Committee on Biological Standardization (the World Health Organization committee commissioned "to establish detailed recommendations and guidelines for the manufacturing, licensing, and control of blood products, cell regulators, vaccines and related *in vitro* diagnostic tests").<sup>13</sup>

The World Health Organization HCV IS has been used as the reference to compare the quantification of HCV genotype panels provided by the NIBSC<sup>14</sup> and regional reference preparations throughout the world.<sup>15</sup> Although the original intended purpose for this IS was for the establishment of sensitivity criteria for molecular assays used to screen blood products in the plasma manufac-

**Table 1.** World Health Organization International Standards for Molecular Infectious Disease Testing

Target	Year established	Assigned value*	Mean assayed value <sup>†</sup>	Characteristics <sup>‡</sup>
HCV RNA First International Standard (96/790)	1997	4.70 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	5.0 log <sub>10</sub> NAT detectable units/ml	HCV-positive donation; genotype 1; diluted in cryosupernatant; freeze-dried <sup>10</sup>
HBV DNA First International Standard (97/746)	1999	5.70 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	6.42 log <sub>10</sub> equivalents/ml	Single donor material, Eurohep R1; genotype A, HBsAg subtype adw2; diluted in pooled plasma; freeze-dried <sup>19</sup>
HIV-1 RNA First International Standard (97/656)	1999	5.00 log <sub>10</sub> IU/vial (reconstituted to 1.0 ml)	4.75 log <sub>10</sub> copies/ml in quantitative assays	HIV PCR-positive/antibody-negative plasmapheresis donation; genotype B; diluted in defibrinated plasma; freeze-dried <sup>18</sup>
HCV RNA Second International Standard (96/798)	2003	4.70 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	5.0 log <sub>10</sub> NAT detectable units/ml	HCV positive donation; genotype 1; diluted in cryosupernatant; freeze dried; separate lyophilization of same material as the first HCV IS <sup>12</sup>
HAV RNA First International Standard (00/560)	2004	4.70 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	5.29 log <sub>10</sub> equivalents/ml	HAV-positive donation diluted in HCV-, HAV-, HIV-, HBV and HBsAg-, anti-HCV-, anti-HIV-negative human plasma <sup>20</sup>
Parvovirus B19 DNA First International Standard (99/800)	2004	5.70 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	5.89 log <sub>10</sub> copies/ml	Parvovirus B19-positive donation; diluted in pooled plasma; freeze-dried <sup>21</sup>
HBV DNA Second International Standard (97/750)	2006	5.70 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	6.30 log <sub>10</sub> equivalents/ml	Single donor material, Eurohep R1; genotype A, HBsAg subtype adw2; diluted in pooled plasma; freeze-dried; second aliquot of original preparation <sup>19</sup>
HIV-1 RNA Second International Standard (97/650)	2006	5.56 log <sub>10</sub> IU/vial (reconstituted to 1.0 ml)	5.35 log <sub>10</sub> copies/ml in quantitative assays; original testing	Field isolate: HIV-1, genotype B env V3, gag; propagated on PBMCs, diluted in pooled human cryosupernatant <sup>18</sup>
<i>Plasmodium falciparum</i> DNA First International Standard (04/176)	2006	8.7 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	8.51 log <sub>10</sub> equivalents/ml	Freeze-dried whole blood patient blood collected by exchange transfusion <sup>23</sup>
HCV RNA Third International Standard (06/100)	2007	4.89 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	Not applicable	Window period anti-HCV-negative plasma, genotype 1a HCV <sup>13</sup>
HPV type 16 DNA First International Standard (06/202)	2008	6.70 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	1 IU/ml is traceable through calculations to 0.947 GEq/ml, determined by independent methods	Freeze-dried recombinant plasmid containing full-length HPV-16 DNA in a background of purified human genomic DNA <sup>22</sup>
HPV type 18 DNA First International Standard (06/208)	2008	6.70 log <sub>10</sub> IU/vial (reconstituted to 0.5 ml)	1 IU/ml is traceable through calculations to 1.031 GEq/ml, determined by independent methods	Freeze-dried recombinant plasmid containing full-length HPV-18 DNA in a background of purified human genomic DNA <sup>22</sup>

HAV, hepatitis A virus; HBV, hepatitis B virus; HBsAg, hepatitis B surface antigen; PMBC, peripheral blood mononuclear cell; HPV, human papilloma virus; GEq, genome equivalents.

\*Concentration is in IU/vial. Some vials are reconstituted only to 0.5 ml.

<sup>†</sup>Assayed value given when determined through global collaborative studies or when assayed by independent methods before assignment of IU.

<sup>‡</sup>Characteristics and instructions for use are at <http://www.NIBSC.ac.uk>, last accessed August 20, 2009.

turing industry, it has contributed to the standardization of blood donation screening assays as well as molecular diagnostic and monitoring assays.<sup>16,17</sup>

The same process has been applied to other World Health Organization IS preparations for nucleic acid testing, and since 1996 standards for HIV,<sup>18</sup> hepatitis B virus,<sup>19</sup> hepatitis A virus,<sup>20</sup> parvovirus B19,<sup>21</sup> human papillomavirus,<sup>22</sup> and *Plasmodium falciparum*<sup>23</sup> have been established. The primary targets have been those most needed to ensure safety of transfused blood and human blood-derived biological products (Table 1).

### *Efforts to Increase the Breadth and Depth of Standards for Quantitative Molecular Infectious Disease Testing*

The traditional intended purpose for international biological standards was to characterize vaccine preparations and establish the analytic sensitivity of assays used to screen blood product materials. This provides insight into the reasons that these materials today are single-point references with no specification for recoverability when

diluted to prepare quantitative standardization curves. Although the establishment of the first World Health Organization HCV IS was consistent with those intended uses,<sup>16,17</sup> the World Health Organization and NIBSC have recognized the role it and subsequent viral IS materials have had in quantitative diagnostics. A 2007 report of World Health Organization collaborating centers for biological standards and standardization describes a 5-year strategic plan “to prioritize the development of World Health Organization biological reference products for the control of blood safety-related IVD [*in vitro* diagnostic] tests,” reiterating the safety of the blood supply as the primary goal.<sup>24</sup> Leaders at NIBSC acknowledge that international standards are needed to standardize quantitative and qualitative molecular infectious disease assays for patient testing and are proceeding to address this area.

Recommendations for the desired specifications of standards for quantitative molecular diagnostics have been communicated to World Health Organization through consultation meetings with the organization and through working groups such as Standardization of Genome Amplification Techniques (SoGAT). The group most persistent in continued discussion of performance specifications for quantitative molecular diagnostic international standards has been the Industrial Liaison Committee (ILC), an organization of molecular assay manufacturers working toward the availability of universally accepted reference standards.

The specific interest of this group has been standards to serve the development of quantitative molecular diagnostic assays used in patient testing. ILC and World Health Organization-sponsored meetings with the World Health Organization, US Food and Drug Administration, and other standards organizations were convened in 1998, 2000, and 2002 to discuss this topic. In addition, ILC has presented regularly at SoGAT meetings. Specifications discussed in these meetings included the establishment of reference materials similar to clinical samples, quantification by methods independent of the current diagnostic testing methods, preparations with concentrations adequate to evaluate the entire expected clinical range, dilution protocols and published dilution recovery expectations, and stability testing (2006).<sup>25</sup>

Studies have shown that for commercial molecular quantitative assays for which international standards have been available (HCV and HIV), variability has decreased significantly since the first generation.<sup>26,27</sup> However, a recent survey of 28 laboratories examining the variability of Epstein-Barr virus (EBV) assays across various viral load concentrations, sample types, and assay platforms demonstrated that significant variation still exists.<sup>28</sup> Results of the survey showed that roughly 50% of all results fell within the “acceptable” parameters of  $\pm 0.5 \log_{10}$  for quantitative nucleic acid test (NAT) assays. Similar results from multicenter comparisons have also been observed.<sup>29</sup> Furthermore, greater variation in results was observed among samples containing virally infected cellular material, suggesting that sample preparation methodology, such as DNA extraction, needs further improvements. Last, variability was significantly higher in

interlaboratory comparisons versus intralaboratory comparisons, strongly suggesting a need for assay calibration to a universally accepted reference standard. This amount of variability poses significant challenges in clinical diagnosis as seen in EBV viral load testing in EBV-related tumors.<sup>29</sup>

A similar study for human cytomegalovirus (HCMV) assays in the United States, Europe, and Canada demonstrated comparable problems. Among 33 laboratories testing a panel of seven constructed and three patient-derived samples, 57.6% of quantitative results were within the range of  $\pm 0.5 \log_{10}$  of expected results. Although the authors attributed the variation to several factors, they concluded that a primary measure toward resolution would be the establishment of an international reference standard.<sup>30</sup>

These observations underscore the need for continued work in providing a more diverse toolbox of international standards and the importance these materials have in patient care. Clinical molecular laboratory directors are now engaged in the discussion of needed diagnostic standards for quantitative molecular assays for infectious diseases. One challenge for IVD manufacturers will be to make available affordable reference materials so that frequent quality control protocols can be implemented in laboratories. The following presents singular and combined efforts by several groups to fill these gaps, including work on synthetic materials by the ILC and by the National Institute of Standards and Technology (NIST) (Gaithersburg, MD) and plans for a broader menu of World Health Organization international standards by SoGAT.

### *Evaluating the Role of Synthetic Materials*

In April 2002, a World Health Organization consultation group was convened to consider the role of international reference materials in viral load monitoring by NAT and to define the characteristics of reference materials appropriate for that purpose. The specifications for an ideal international standard and what might be acceptable in light of the diverse challenges of producing and sustaining them for molecular infectious disease testing were discussed. In this meeting, HCV and the viral assays were the predominant focus as the World Health Organization HCV IS had recently been established, although many of the issues were transferrable to other infectious disease targets. Table 2, outlining desired characteristics of molecular standards and the technical and strategic challenges in producing them, is adapted from presentations and deliberations at the meeting and continued discussions into the present (Report: World Health Organization Consultation on International Standards for *in Vitro* Clinical Diagnostic Procedures based on Nucleic Acid Amplification, [http://www.who.int/blood\\_products/publications/en/BIVD02apr22.pdf](http://www.who.int/blood_products/publications/en/BIVD02apr22.pdf), last accessed August 25, 2009). These issues continue to confront organizations attempting to establish globally recognized standard materials.

Because HCV cannot be propagated and measured by independent methods such as limiting dilution viral

**Table 2.** Optimal Specifications and Challenges: Molecular Standards

Characteristic	Optimal specification	Acceptable specification	Challenges
Target nucleic acid	Whole genome as found in virus; intact	Whole genome as found in virus; intact	Is there a representative consensus genome?
Source of target nucleic acid	Virus	Recombinant DNA (DNA viruses) or RNA synthesized <i>in vitro</i> (RNA viruses)	Can the virus be propagated? Is the cultured strain representative?
Length of target nucleic acid	Entire genome	All of the sequences recognized by current and projected future assays	Current assays may not be representative of future technologies Cultured organisms, purified NA or synthetic NA may not have important secondary structure
One or several genotypes/subtypes	All relevant genotypes available as standards	One genotype for a "foundation" standard. Genotypes and subtypes calibrated from the foundation standard	One representative organism does not necessarily address technicalities of all variants  Resources to produce and characterize all variants and perform the global studies to value-assign them would detract from producing standards for more organisms
Which genotype/subtype?	The most globally prevalent	The most globally prevalent	The most globally prevalent or the target of most present and near future testing?
Sequence of target nucleic acid	Known	Known	The proposed material should be sequenced
Concentration of target nucleic acid	Sequence of cDNA clones (from RNA viruses) or DNA clones (from DNA viruses)	Sequence of cDNA clones (from RNA viruses) or DNA clones (from DNA viruses)	Some source material (patient plasma) may not have high enough titers to produce enough standard material.
Matrix	High enough to evaluate calibrators that cover levels of targets found in patients	High enough to evaluate calibrators that cover levels of targets found in patients	Is pooled material appropriate? Can the bodily fluid from one individual be representative? Are decalcified, defibrinated materials commutable? If the infectious target is tested from cells, should cellular standards be developed?
Temperature of storage	Natural material from which the target is predominantly assayed. (ie, blood-borne viruses: plasma)	Material that will ensure stability and intactness of target nucleic acid and that will not affect assay	Can impact stability
Manufacturing process	Ambient or refrigerated Validated, reproducible methods within a validated quality system	Ambient or refrigerated Validated methods within a validated quality system	Could limit the number and type of organizations able to produce or contribute materials
Vial-to-vial consistency	$SD \leq 0.15 \log_{10}$ copies/ml (coefficient of variation $\leq 35\%$ )	SD in target concentration known and published	Could limit the number and type of organizations able to produce or contribute materials
Initial quantification of nucleic acid targets	Independent analytical method(s)	Quantitative NAT using statistically designed protocol and equal weighting of results from assays used	Absence of reference methods requires testing with relevant available methods Are resources available to adequately test materials with an even representation of the available test methods?



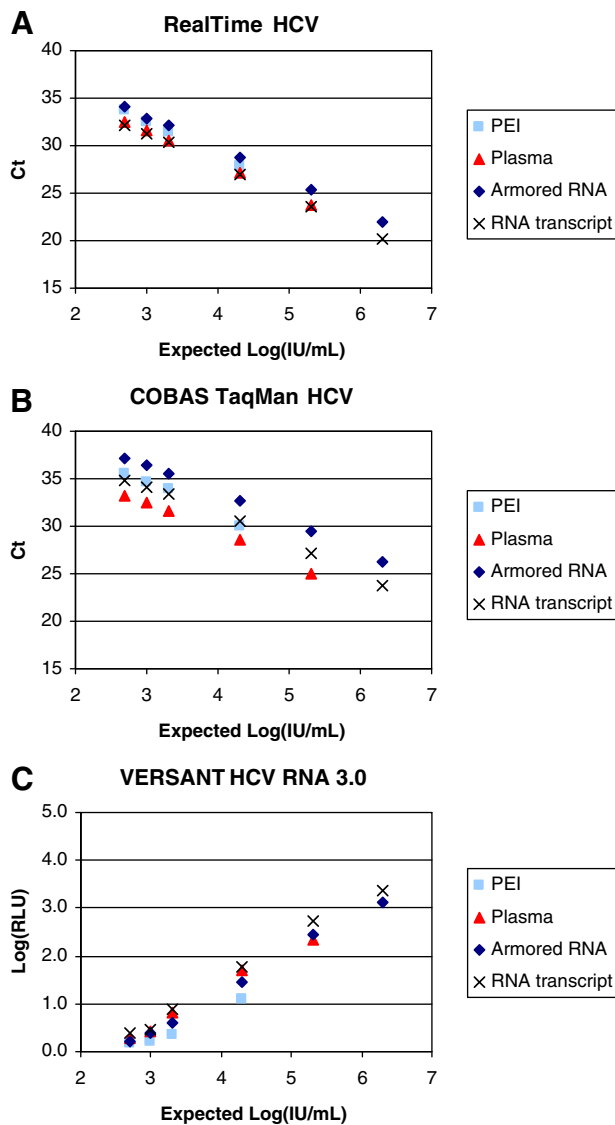
culture, participants at the 2002 meeting designated it as a model for work to explore alternative strategies. Further, considering the desired characteristics discussed, the group concluded that synthetic (*in vitro* synthesized) nucleic acids could be considered. The methods for producing these synthetic materials were not specified at the time; however, a well-characterized, *in vitro*-generated HCV RNA transcript, quantified by physicochemical assays to a primary phosphate standard was readily available for evaluation through a donation from Bayer Diagnostics (now Siemens Healthcare Diagnostics, Inc., Deerfield, IL).<sup>31</sup> The transcript material included target sequences for all of the technologies available.

At the meeting, the ILC was challenged with planning and performing a feasibility study using a synthetic RNA containing HCV sequences, and a series of studies among manufacturers were initiated. The first feasibility study was performed in 2002. Four NAT methods were included in the study: VERSANT HCV RNA 3.0 Assay (bDNA) (Siemens Healthcare Diagnostics, Inc., Deerfield, IL); COBAS AMPLICOR HCV MONITOR, version 2.0 (Roche Molecular Diagnostics, Pleasanton, CA); an internal-use quantitative HCV TMA assay (Gen-Probe, Inc., San Diego, CA); and LCx HCV RNA Quantitative Assay (Abbott Laboratories, Abbott Park, IL). The original concentration of the synthetic HCV RNA was determined to be traceable to a chemical standard (potassium phosphate) by non-NAT methods.<sup>31</sup> Scientists at each site used the synthetic RNA transcript to quantify HCV RNA concentrations in members of a patient-derived panel. Feasibility was defined as a demonstration of panel quantification value agreement within 1.0 log<sub>10</sub> regardless of the NAT method. Values reported by each site as synthetic RNA transcript copies per ml did not differ by more than 1.0 log<sub>10</sub> regardless of the technology used for analysis and demonstrated the feasibility of a synthetic HCV RNA standard. Further studies in 2005 and 2006 by this group evaluated the synthetic material using automated sample preparation instruments to determine that the material could be recovered in full extraction processes. Acceptable recovery was demonstrated; however, handling the RNA transcripts to maintain their integrity required expertise that is not universally available. The results of these studies were presented at the SoGAT meeting in 2004.

The ILC continued this work in 2007 with two synthetic and two biological materials. Scientists from Roche Molecular Diagnostics, Siemens Healthcare Diagnostics, Inc. Berkeley, CA and Abbott Molecular Inc. (Des Plaines, IL) quantitatively tested dilution panels of these materials in their respective HCV assays: Roche COBAS AmpliPrep/COBAS TaqMan HCV Assay, Siemens VERSANT HCV RNA 3.0 Assay (bDNA), and Abbott RealTime HCV assay. The synthetic materials were the HCV RNA transcript supplied by Siemens Healthcare Diagnostics, Inc. (described previously) and an HCV Armored RNA Quant supplied by Asuragen, Inc. (Austin, TX). Both materials had been quantified relative to a phosphate standard by independent, non-NAT methods. The Armored RNA, an RNA transcript synthesized by *in vitro* transcription, is encapsulated in a bacteriophage protein coat and is stable to nucleases present in normal sample matrices. It

provided a material that could be subjected to the entire assay procedure without special handling. The biological materials used were the Paul Erlich Institute regional HCV standard at 80,000 IU/ml, contributed by M. Nübling at the Paul Erlich Institute (Langen, Germany) and an unquantified high titer plasma material prediluted into negative plasma prepared by scientists at Abbott Molecular, Inc. Six to eight replicates of four to six linear points of each material were tested in each assay in the manufacturers' laboratories. The synthetic materials demonstrated full utility based on recovery, range of linearity, and variation. In all three assays, the synthetic targets could be recovered and quantified with equal or less variability than the biological materials (Figure 1). The study, findings, and suggestions of possible roles of both synthetic and biological standards were presented at the SoGAT meeting in June 2007.

Because the purpose of international standards is to provide the initial calibration point for developing assays and subsequent versions of those assays, it is critical that the standard remain reliable and unchanged. This is most challenging for biological standards in a field of evolving technologies. Each time a replacement World Health Organization International Standard is assigned a value, several years have passed, and the technologies that were used to evaluate the previous standard may not exist. Even when the former and latter are tested in unison, there may be small shifts in the valuation of the established standard, as described earlier in the World Health Organization HCV IS replacement study and documented in the HIV IS replacement studies. The impacts of these shifts are challenging to project and impossible to measure over time because of the limited amount of World Health Organization material available. For the World Health Organization global collaborative studies described in this review, four vials of candidates per method tested are available to the participants, and when the standard is established, all those requesting the material for development purposes are limited to a specific number of vials per year. This limitation is necessary so that the maximum distribution can be provided but has the unintended consequence of prohibiting statistically relevant longitudinal studies of the impact of small changes in the standards. To mitigate the resource limitation, there are several national and regional standards and secondary reference materials calibrated from the World Health Organization International Standards. In addition, diagnostic test manufacturers have procedures for developing secondary, internal use references calibrated against and traceable to the World Health Organization International Standards. Clinical scientists constructing laboratory developed tests are expected to act in a similar manner; either by generating or purchasing secondary reference materials for their internal development and calibration activities. However, with each dilution of the IS and calibration of secondary materials, there is inherent error as demonstrated in ISO 17511:2003. Because the molecular community may not be able to adequately project or quantify this impact, it is even more critical that we contribute to support, critique, and maintain the integrity of continued replacement of biological standards.



**Figure 1.** Evaluation of synthetic and biological HCV reference materials with three commercial HCV quantitative assays. Serial dilutions of the Paul Erlich Institute (PEI) regional HCV standard (80,000 IU/ml), high titer plasma, Armored RNA Quant HCV and synthetic HCV RNA transcript were tested using the RealTime HCV (A), COBAS AmpliPrep/COBAS TaqMan HCV (B), or VERSANT HCV RNA 3.0 assays (C). All test specimens were shipped and stored below  $-70^{\circ}\text{C}$ . The HCV Armored RNA was pre-diluted, shipped, and stored in base human serum. Other specimens were formulated by each laboratory using the same base matrix immediately after thawing of the stock materials. The synthetic HCV RNA transcript was diluted in the presence of lysis reagent to inhibit potential degradation of the RNA by serum nucleases. At least six replicates of each dilution were tested in three independent runs with each assay. The synthetic materials were formulated in copies/ml of base matrix ( $2.5 \times 10^3$ ,  $5 \times 10^3$ ,  $1 \times 10^4$ ,  $1 \times 10^5$ ,  $1 \times 10^6$ , and  $1 \times 10^7$  copies/ml) and converted to IU/ml (IU/ml = copies/ml  $\times$  5). Ct, threshold cycle; RLU, relative light units.

To contend with these issues and the limitations of the materials, a scenario was proposed whereby well characterized and independently tested synthetic nucleic acid material could serve as the constant in an environment in which reference methods are not available. If stable synthetic materials could be established along with the international biological standard and the relationship between the two could be quantified for relevant technologies, the synthetic material could be tested with

each replacement lot of the biological standard. Test developers would continue to use the biological standards, but each replacement lot of those materials would be tested against the “unchanging” synthetic material. Because of the potential greater supply of synthetic materials, they could also be used in the characterization of secondary reference materials. This concept has not been tested for molecular diagnostics; however, current development of an HCMV nucleic acid material at the NIST and a biological standard for NIBSC provides a timely opportunity.

### Development of a HCMV Standard Reference Material at NIST

Because HCMV resides intracellularly and is also present in plasma, this virus and those like it present an added level of complexity for producing standards: determining the biological matrix that most universally represents what is tested in clinical practice. Considering this, a standard that is free of biological material may be an appropriate reference for the development of several types of biological standards. The NIST has undertaken a project to develop a standard reference material for human cytomegalovirus. The material will consist of pure HCMV DNA. The goal is to develop a reference that would be traceable to the SI (International System of Units, the metric system) and suitable as a calibrant for quantitative PCR assays of HCMV viral load but perhaps more importantly as a tool to standardize various NAT assay reagents and calibrants produced by manufacturers of diagnostics or laboratory developed protocols.

Quantitative real-time PCR is rapidly becoming the method of choice for HCMV quantification over previously developed assays such as antigenemia because of its sensitivity and speed<sup>32</sup> and the possibility of high throughput implementation. But there are issues such as DNA sequence diversity among targets used for quantification that can result in false-negative results.<sup>33,34</sup> There is a proliferation of assays with different target sequences in the HCMV genome. In a recent survey, 10 different open reading frame targets were used, glycoprotein B being the most frequent.<sup>35</sup>

The HCMV standard reference material will consist of pure DNA from a Towne strain HCMV bacterial artificial chromosome (BAC).<sup>36</sup> The BAC DNA insert is 10 kbp and replaces the US1 to US12 open reading frames in the Towne cytomegalovirus DNA by homologous recombination. The final construct,  $\Delta_{UL147}$  Towne BAC, will be used to produce viral DNA.<sup>37</sup> The Towne BAC is stable and can provide large quantities of consistent viral DNA, whereas cultured virus, especially from laboratory strains of HCMV, can produce truncated genomes.<sup>38</sup> Genome size consistency is important for calculation of genome copy number.

The HCMV Towne BAC DNA reference material will be certified for sequence. The intent is to sequence all of the relevant regions of the genome that are used as targets for PCR assays. Two approaches will be used for quantification of the BAC DNA. Digital PCR is a primary

method for counting copies of genomes or plasmids. This approach could provide traceability to the mole. Pure DNA can be accurately and precisely quantified based on phosphorus measurements using high-performance inductively coupled plasma-optical emission spectroscopy.<sup>39</sup> Once the HCMV standard reference material has been certified, interlaboratory studies will be conducted.

### *SoGAT Committee for Viral Diagnostics*

Originally sponsored by the World Health Organization to address the safety of transfused blood, tissue, and organs, with emphasis on viral, bacterial, and parasitic contamination, SoGAT has convened at least annually for more than 12 years. NIBSC has maintained leadership of this group with representatives from public health and other government agencies, IVD and independent control manufacturers, and diagnostic and university laboratories. The meeting provides a forum for discussion on assays, reference materials, and regulatory issues as well as for organizing collaborative studies. At the SoGAT meeting in June 2007, it was announced that a second series of meetings would be started with the focus on molecular diagnostics for clinical virology. The goals for the new group will be patterned on those for the original group, and it is expected there will be crossover interests between the two groups. SoGAT-CD (SoGAT Clinical Diagnostics) convened for the first time in June 2008.

At the first meeting of SoGAT-CD, issues specific to molecular testing for infectious diseases were discussed. The lack of quantitative reference materials for the growing number of viral molecular tests was a persistent concern. Within months of the meeting, the NIBSC issued draft protocols for comment regarding the establishment of two reference materials: one for HCMV and one for EBV quantitative assays. At the next meeting, in September 2009, participants discussed the progress and path forward of the CMV and EBV standards.

### *The Joint Committee for Traceability in Laboratory Medicine*

The JCTLM was formed in 2002 with a Declaration of Cooperation between the International Committee of Weights and Measures, the International Federation of Clinical Chemistry and Laboratory Medicine, and the International Laboratory Accreditation Cooperation and is sponsored by the International Bureau of Weights and Measures. The stimulus for this committee came from the IVDD, Directive 98/79/EC of the European Parliament and of the Council of October 27, 1998, on *in vitro* diagnostic medical devices. The work of the committee is to facilitate the implementation of traceability to higher order reference materials and measurements that are required by the IVDD. Committee participants include national measurement institutes of several countries, other government agencies (such as US Centers for Disease Control and Prevention), and industry and laboratory associations.

Global standardization can lead to the ultimate goal of achieving correct and equivalent measurements on patients' samples anywhere, at any time, and independent of specific assay. Different assays should be able to produce comparable results with consistent uncertainties and intervals. As an important step in that direction, there needs to be higher-order reference materials and reference measurements. The term "higher-order" was not defined in the IVDD; but the requirements are described in ISO 15193 and ISO 15194.<sup>40,41</sup> The JCTLM Working Group 1 evaluates submitted nominations for reference materials and methods by determining compliance with these ISO standards. Based on this, two lists are published in the JCTLM database. List 1 describes certified reference materials and measurement methods that are traceable to the SI. The kinds of materials include small molecules, such as electrolytes, metabolites, and some proteins. List 2 includes reference materials, not traceable to the SI, but traceable to an internationally agreed on protocol. Examples here are coagulation factors or blood typing. Working Group 1 is divided into review teams for nine categories of analytes, including nucleic acids. Announcements for new nominations to be considered for inclusion on the lists are made periodically, and listed materials are removed when they are no longer available. The JCTLM database is hosted on the International Bureau of Weights and Measures website, where documentation to provide transparency to the decision-making process is available (Joint Committee for Traceability in Laboratory Medicine, <http://www.bipm.org/en/committees/jc/jctlm>, last accessed August 26, 2009). Armbruster and Miller<sup>42</sup> have written a comprehensive review article on JCTLM and its role in global standardization.

The JCTLM Working Group 1 Nucleic Acid Review Team has experts from Japan, Europe, and the United States representing bioindustry, molecular diagnostics manufacturers, reference material producers, and public health organizations. The group is preparing the criteria that will be applied to assess the "quality" and "higher-order traceability" of JCTLM database-nominated nucleic acids reference materials, which only have stated "nominal qualities," most usually sequence. There is limited ISO standard guidance on the criteria that need to be fulfilled to consider a reference material's nominal properties as those of higher-order or high-quality (although issues of identity, stability, homogeneity, and commutability need to be considered). Developing clear guidance on internationally accepted criteria against which the traceability of nucleic acid reference materials can be reviewed will hopefully encourage the nomination of more nucleic acids reference materials to the JCTLM database.

### *Association for Molecular Pathology*

The Association for Molecular Pathology is a not-for-profit scientific society dedicated to the advancement, practice, and science of clinical molecular laboratory medicine and translational research based on the applications of genomics and proteomics (Association for Molecular



Pathology, <http://www.amp.org>, last accessed August 27, 2009). The Association for Molecular Pathology Clinical Practice Committee Working Group on Infectious Disease has focused on the need for standardization of quantitative molecular viral load testing. Quantitative PCR procedures, in particular real-time PCR, are popular ways to monitor viral load because of the reliability and broad linear range of quantification. Many different assays have been developed, often in individual laboratories. The need for reference standards for normalization of laboratory measurements is continually recognized and emphasized by the Association for Molecular Pathology in meetings, workshops, discussion threads, and collaborative studies.<sup>28,29,32</sup> The organization is working with the NIST to produce an HCMV Standard Reference Material. Future projects include assessing the need for reference standards for quantitative BK virus and EBV viral load testing.

### **Perspective and Future Issues**

The benefit of having internationally recognized reference materials for quantitative molecular tests is now well recognized by all of the constituencies involved in development and testing: clinical laboratories, independent control and reference material manufacturers, IVD manufacturers, and regulatory and policy makers. Challenges now involve communication of the complex issues across these diverse stakeholders and exploring different approaches to strategic, technical, and applications issues. Strategic issues include agreement on target priorities and design and management of studies. For technical issues, linearity of the materials, the relationship of synthetics to biological products, biological matrices, possible standard reference methods, and commutability are topics that are surfacing.

Until recently, the impact of commutability on standardization of laboratory results has not been adequately stressed. A review in 2006 of the Joint Committee for Traceability in Laboratory Medicine's list of approved reference materials revealed very few that were validated for commutability against native clinical samples.<sup>43</sup> One of the most important considerations in the development of effective standards and reference materials is that the material be commutable, ie, behave as closely as possible to the test samples in the full range of measurement procedures expected to make use of the material. Commutability is described as equivalence of the mathematical relationships, or ratios, between the results of different measurement procedures for a reference material and for test samples of normal and pathological origin.<sup>44</sup>

Commutability does not apply solely to synthetic reference materials. Biological standards are often assumed to be commutable because of their native origin but may not have been specifically validated as such. In 2005 a committee was formed by the Clinical and Laboratory Standards Institute for the development of guidelines to establish consistent assessment of commutability of refer-

ence materials.<sup>45</sup> Application of commutability to standards and secondary reference materials is expected to be a seminal topic for diagnostics in the future and molecular diagnostics will necessarily be part of that discussion.

Other important technical issues include understanding the relationship between the different approaches to standards that technological advancements may provide in the near future. Plans for the establishment of both synthetic and biological standard materials for HCMV have already been discussed in this review. Experiments to understand the relationship of each of these materials to each other and the role each will play in standardization of HCMV assays will include discussions of traceability, commutability, and strategy.

The recent interest from all levels of stakeholders in the effort to establish international standards for quantitative molecular diagnostics will lead to further inclusivity, transparency, and critical evaluation. It will be important to maintain avenues of communication that have opened up through professional organizations such as Association for Molecular Pathology, European Society for Clinical Virology, and Pan American Society for Clinical Virology. The ILC has reaffirmed its primary objective to work in the area of these technical and strategic issues regarding the establishment and replenishment of international standards and is encouraged by the significant attention being given to these critical issues by others. Discussion, critique, and constructive efforts specific to the issues in quantitative molecular diagnostic standards are really just beginning. Continued and active involvement of all stakeholders will be necessary to achieve the common goal of reliable and commutable patient results in molecular infectious disease testing.

### **Acknowledgments**

We acknowledge Jan Turczyn and the scientists at Siemens Healthcare Diagnostics (Berkeley, CA) for their contribution of HCV standard materials and their participation in the synthetic materials feasibility experiments. We also acknowledge Micha Nübling and the Paul Erlich Institute for the contribution of the HCV Paul Erlich Institute biological standard reference used in the synthetic materials feasibility experiments. Finally, we acknowledge the framework and support of the Industrial Liaison Committee and the chair, Jim Reid. The need for this review was recognized, formed, and supported through the organization's discussions, workshops, and experimentation. Particular thanks are given to Giuseppe Colucci for editorial input.

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