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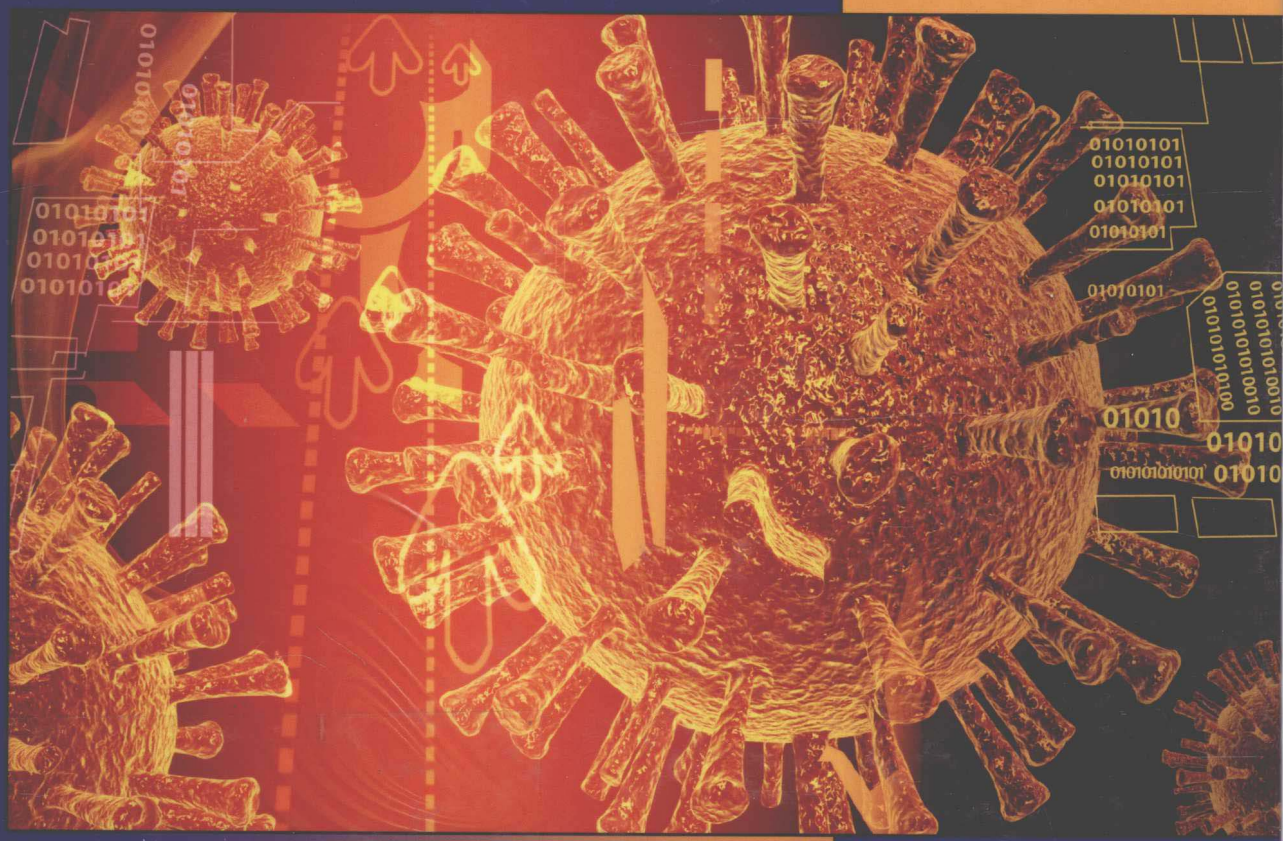
Volume 50

Lennette's Laboratory Diagnosis of Viral Infections

Fourth Edition



Edited by
Keith R. Jerome

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Edwin H. Lennette: A Tribute

Ed Lennette was born in Pittsburgh, Pennsylvania, on September 11, 1908, and died of respiratory failure on October 1, 2000, following surgery. These cold, hard facts in no way describe or tell us anything useful about this man and his accomplished life.

He earned a B.S. degree at the University of Chicago in 1931 and a Ph.D. degree in 1935. His Ph.D. degree is believed to be the first awarded specifically in the field of virology. Ed then completed an M.D. degree at Rush Medical College (also at the University of Chicago) in 1936, and following his internship, he spent brief periods at the Pathology Department of Washington University School of Medicine in St. Louis, Missouri, and at the Rockefeller Foundation laboratories in New York City.

At that time the Rockefeller Foundation was interested in yellow fever and its International Health Division (IHD) assigned him to Brazil, where he spent most of World War II, working on yellow fever and encephalitis viruses.

In 1944, the IHD transferred him to their laboratory in Berkeley, California, to work on hepatitis and encephalitis. When that laboratory was transferred to the California Department of Public Health in 1947, Ed became its Director, following a year as Chief of the Medical-Veterinary division of the U.S. Army facility at Fort Detrick, Frederick, Maryland.

For the next 31 years he molded this laboratory into a world-renowned training laboratory, as well as a, perhaps the, leading laboratory for the diagnosis of viral and rickettsial diseases. This Viral and Rickettsial Diseases Laboratory, or VRDL as it was known, conducted substantial programs of research on Q-fever and arthropod-borne encephalitis, on polioviruses and other infections, and on the role of viruses in causing human cancer. Many of the people trained at the Berkeley VRDL went on to become leading scientists and administrators of laboratories and health agencies worldwide. He also served as a consultant to many government agencies and participated in numerous advisory committees.

Ed published many scientific papers and edited several books that became classics in their field. Among them was "Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections," coedited with Drs. Nathalie J. Schmidt and Richard W. Emmons.

After he retired from public service in 1978, Ed became the President of the California Public Health Foundation, and in 1981 and 1982, served as Acting Director of the W. Alton Jones Cell Science Center in Lake Placid, New York.

Of course, I had heard of Dr. Lennette and his legendary accomplishments, but it was only in the 1980s that we met for the first time, although I had participated in writing chapters for earlier editions of his book on diagnostic virology. Awed by meeting such a senior person and eminent scientist, Ed put me at ease immediately. To this day, I remain in awe of him.

His encyclopedic recall and brilliant, practical insights were remarkable. To the end he strongly supported gaining experience that leads us to knowledge, as opposed to exclusively technical procedures, relied on by some in the rapidly moving field of diagnosis. Whenever, as Moderator for Virus Diseases of ProMED-mail, I would comment harshly (but fairly!) about an organization misinterpreting or overinterpreting its data and suggest to them a different, albeit "old-fashioned," method, or if I simply said someone was wrong, I could expect a telephone call or e-mail from Ed saying, essentially, "Right on, baby."

At least as much as I enjoyed hearing about his experiences and rereading his early papers with Hilary Koprowski, Bill Hammon, and many more of the founding fathers of virology, I enjoyed his company. Ed was tough; he didn't take any guff from anyone, although he usually was diplomatic and always polite. He had a marvelous sense of humor, and he was socially adventurous, generous of his time, and patient with young people. Through sorrows and disappointments, Ed Lennette maintained his love of life. He was one of the great people in my life and in the lives of many, many others, and his influence lives on, as witness this book.

Charles H. Calisher

Preface

One of the great joys of editing this latest version of *Laboratory Diagnosis of Viral Infections* has been learning more about the original editor, Dr. Edwin H. Lennette. Although I never had the privilege of meeting Ed, the stories and anecdotes cheerfully offered by his many friends and colleagues have made me feel as if I knew him. At the same time, this has presented a problem—to try to meet the impossibly high bar Ed set as an editor who also had a distinguished career as a scientist and diagnostician. Although it would be futile to try to fill Ed's shoes, I hope that this text fulfills the promise to inform the field he loved so well. To honor Ed, then, it is altogether fitting that this series has been renamed *Lennette's Laboratory Diagnosis of Viral Infections*.

A major challenge in assembling this text was finding a niche not already occupied by any of the other excellent books touching upon diagnostic virology. This inspired a significant reorganization. As in previous editions, the work is divided into two parts. Part one is similar in scope to that of the previous editions and provides a detailed description of the various techniques forming the foundation of modern diagnostic virology. Part two, on the other hand, presented a greater challenge. A simple listing of virus families and their various clinical manifestations was clearly the easiest option, but this approach has already been well traveled. Instead, we have taken a syndromic approach, an idea originally suggested by my colleague, Dr. Yi-Wei Tang. Thus, if presented with a patient having symptoms of viral encephalitis, for example, readers can now refer to the chapter on CNS infections, where they will find a differential diagnosis of potential causative agents, along with suggestions for the appropriate diagnostic approach. While this reorganization has brought its own challenges in avoiding redundancy and omissions, I believe this unique approach will make the book particularly valuable to students of infectious disease as well as laboratorians.

Clinical virology has changed at an astounding pace in the 10 years since publication of the previous edition, and this edition has been completely rewritten to reflect this new reality. Molecular techniques continue to grow in importance and are covered in depth by new chapters on a variety of topics, including the design of molecular tests, the importance of genotyping and viral sequence analysis, and the use of microarrays in diagnostic virology. Another emerging theme is the increased awareness of global health issues, reflected here by a new chapter regarding viral testing in resource-limited settings. Finally, new associations continue to be made between clinical disease and viruses, and these are discussed in the chapters on respiratory infections, polyomavirus infections, hemorrhagic fevers, and elsewhere throughout the book.

The process of bringing this edition to reality owes much to Maria Lorusso at Informa, who initially brought the project to my attention, and Aimee Laussen, also of Informa, who has taken care of innumerable logistical issues since the early days of the project. I would also like to thank my colleague, Dr. Rhoda Morrow, for advice and support at many stages along the way.

In his preface to the first edition, Ed stated that the book was directed toward the laboratorian who needs a ready reference source to assist in reaching a laboratory diagnosis of a viral infection. This remains the goal of the new edition; no easy task given the rapid changes in technology, the continuing emergence of new viruses, and newly described viral etiologies for clinical syndromes. I hope that readers of this new edition will find the book useful and will gain a little of Ed's enthusiasm for this ever changing and endlessly fascinating field.

Keith R. Jerome

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Contents

1 Verification and Validation of Virological Laboratory Tests in the Routine Diagnostic Laboratory

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INTRODUCTION

Routine viral diagnostics includes techniques for both indirect and direct detection of viruses. Indirect detection of viruses is performed by serological studies. Techniques for direct detection of viruses include detection of viral antigens, viruses, or viral components by isolation of viruses on cell cultures (or through animal experiments), and detection of viral nucleic acids is also referred to as nucleic acid testing (NAT). Furthermore, viral morphologic structures can be investigated by means of transmission electron microscopy.

Today, NAT is having a major impact on viral diagnostics. Molecular assays are used in many if not most virological laboratories. Technological improvements, from automated sample preparation to real-time amplification technology, provide the possibility to develop and introduce assays for most viruses of clinical interest. The risk of contamination has been reduced significantly and the turnaround time to generate results shortened. In contrast, standardization and quality assurance/quality control issues have often remained underemphasized, requiring urgent improvement.

Moreover, it must be taken into consideration that reliable viral diagnostics depend on additional preanalytical issues, such as choice of the correct sample material, optimal sampling time with regard to the course of disease, and the duration and conditions of sample transport to the laboratory.

QUALITY ASSURANCE/QUALITY CONTROL

In the international standard ISO 15189, special requirements for medical laboratories have been established. Among several issues, this standard demands certain verification and validation procedures. For laboratories in the United States, the FDA has established regulations based on existing ISO standards (1).

The European Union's Directive on In Vitro Diagnostic (IVD) Medical Devices (98/79/EC) requires data demonstrating that an IVD achieves the stated performance and will continue to perform properly after it has been shipped, stored, and put to use at its final destination (2).

Quality control systems have been implemented in the majority of routine diagnostic laboratories. In contrast to certification that is mainly based on the supervision, description, and conformity of processes, accreditation additionally focuses on the competence of the laboratory providing reliable test results and their correct interpretation.

Quality assurance requires careful documentation in the routine diagnostic laboratory. For each newly implemented test or test system, a standard operating procedure must be available. Additionally, verification or validation data must be available for each test.

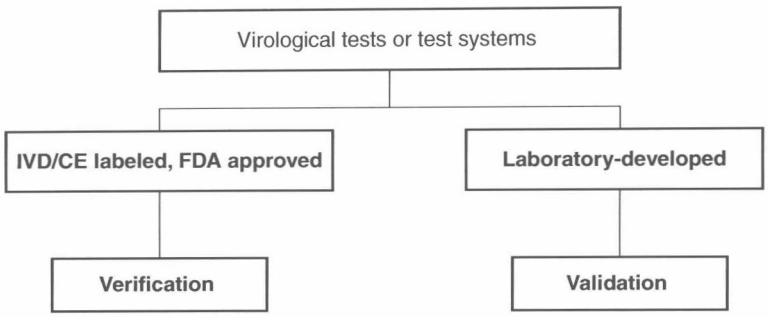


Figure 1 Verification/validation of virological tests or test systems.

Verification and Validation of Tests or Test Systems Employed in the Routine Laboratory

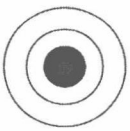
Suitability of a technique does not necessarily mean that it is performed correctly and provides valid results. The ISO 15189, the IVD Directive 98/79/EC, and the FDA regulations (clearly described in the *Code of Federal Regulations*) require verification or validation of each investigational procedure in order to prove both the correct application and the correct performance of a diagnostic test. The complexity and the extent of the verification or validation procedure depend on whether an IVD/CE-labeled and/or FDA-approved test or a "home-brewed" laboratory-developed test or test system is involved. For a laboratory-developed test or test system, "analyte-specific reagents" (ASRs), medical devices that are regulated by the FDA, should be used preferentially. Implementation of any reagent labeled "research use only" (RUO) is not permitted for any test or test system in the United States routine laboratory. In Europe, one or more RUO reagents may be implemented following validation of the test or test system. Both terms "in vitro diagnostic medical device" as used in the IVD Directive 98/79/EC and "device" as used in the FDA regulations do not only mean "test" but also "test system" if more than a single component is required to generate a diagnostic result. For instance, molecular test systems based on PCR usually consist of a combination of different reagents and instruments for nucleic acid extraction, amplification, and detection of amplification products.

Verification or validation work has to be done if a new test or test system is introduced in the routine diagnostic laboratory (Fig. 1). Additionally, any change of an existing test procedure requires further validation work (3).

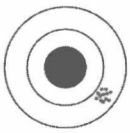
For a commercially available IVD/CE-labeled and/or FDA-approved test or test system, the manufacturer is responsible that the IVD achieves the performance as stated. Nevertheless, the user must verify that performance characteristics, such as accuracy and precision, are achieved in the laboratory (Table 1). The accuracy (or "trueness" in the recent nomenclature) is defined as the degree of conformity of a measured or calculated quantity to its actual (true) value and can be estimated by analyses of reference materials or comparisons of results with those obtained by a reference method (Fig. 2). These are the only accepted approaches to trueness. When neither is available, other evidence is required to record the ability of the method to measure the analyte. The imprecision is defined as the level of deviation of the individual test results within a single run (intraassay imprecision) and from one run to another (interassay imprecision) (Fig. 2). Imprecision is usually characterized in terms of the standard deviation of the measurements and relative standard variation (variation coefficient). In case of a

Table 1 Minimum Requirements for Verification or Validation of a Test or a Test System in Clinical Virology

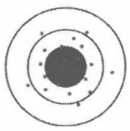
Verification	Validation
Accuracy	Accuracy
Imprecision (intra- and interassay)	Recovery
Linearity (if quantitative)	Selectivity
	Imprecision (intra- and interassay)
	Linearity (if quantitative)



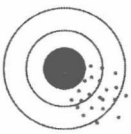
Accurate and precise



Inaccurate but precise



Imprecise but accurate



Imprecise and inaccurate **Figure 2** Accuracy ("trueness") and imprecision.

quantitative test or test system, the linearity must be evaluated additionally (Table 1). The linearity is defined as the determination of the linear range of quantification. Data for linearity studies should be subjected to linear regression analysis with an ideal regression coefficient of 1. In case of a nonlinear curve, any objective, statistically valid method may be used (4).

In contrast, the clinical laboratory that uses laboratory-developed test or test systems, or combines different IVD/CE-labeled and/or FDA-approved tests or test systems without recommendation of the manufacturer, is acting as manufacturer of a medical device and thus responsible for both the suitability and the correct performance of the test. Those tests or test systems must be validated including accuracy, recovery, selectivity, imprecision, and, if quantitative, linearity (Table 1). Recovery (also known as "analytical sensitivity") studies involve analyses after known amounts of analyte are added to the biological matrix on which the determination will be performed. Selectivity (also known as "analytical specificity") testing reflects the ability of an analytical method to detect an analyte (and quantify it in case of a quantitative test or test system) in complex mixtures of biological sample material also referred to as matrix. For selectivity testing, cross-reactivity with any other analyte has to be excluded. Furthermore, interference studies must be performed to assess the effects of possible interferents including, for instance, hemoglobin, rheumatoid factor, and autoantibodies, and those of exogenous materials, such as ingredients of blood collection containers and commonly used or coadministered drugs. It is important to mention that the introduction of an internal control (IC; see below) checks for a possible matrix-induced effect and ensures the reliability of a NAT test or test system.

Minimum requirements for verification and validation procedures for virological tests or test systems are described in the following sections. A more simplified validation procedure may be applied if calibrators are not commonly accessible or if a test or test system for validation is based on a scientific publication. In general, reference material, patient samples, or pooled sera may serve as calibrators for a verification or validation experiment. If patient samples or pooled sera are used, they must have been tested earlier with the existing "gold standard," as far as available and/or defined. Calibrators are classified into positive, low-positive, and

negative controls. For detection of virus-specific antibodies and viral antigens, positive controls are defined as having concentrations more than threefold above the limit of detection (LOD; see below) or the limit of quantitation (LOQ; see below) of the test or test system, and within the upper limit of linearity, while low-positive controls are defined as having concentrations up to threefold over the LOD or the LOQ of the test or test system. For NAT, positive controls are defined as having concentrations more than 1 $\log_{10}$ over the LOD or the LOQ of the test or test system and within the upper limit of linearity for detection of virus-specific antibodies and viral antigens, while low-positive controls are defined as having concentrations up to 1 $\log_{10}$ over the LOD or LOQ of the test or test system. If more than one positive control is necessary to complete testing for certain performance characteristics, they should always contain different concentrations (within the linearity range as defined above) of the parameter to be tested.

Minimum requirements outlined in this chapter are valid for all verification and validation procedures in clinical virology. However, tests or test systems for pathogens included in List A of Annex II to Directive 98/79/EC (human immunodeficiency virus type 1 and 2, human T-cell lymphotropic virus type I and II, hepatitis B, C, and D viruses) are not covered here because of special regulations (Directive 98/79/EC, Article 9). Common technical specifications enforced for tests or test systems on those parameters are outlined in the Commission Decision of May 7, 2002, on common technical specifications for IVD medical devices (5).

Minimum Requirements for Verification of IVD/CE-Labeled and/or FDA-Approved Tests or Test Systems for Detection of Virus-Specific Antibodies, Viral Antigens, or NAT

If a new IVD/CE-labeled test or test system for detection of virus-specific antibodies, viral antigens, or NAT is introduced in the routine diagnostic laboratory, verification experiments are performed to verify accuracy, imprecision, and, in case of a quantitative test or test system, linearity (Table 2). For determination of the accuracy, three positive, three low-positive, and three negative samples are used. In case of a qualitative test or test system, one positive and one low-positive sample are used for determination of intraassay imprecision. Each sample is tested three times within a run. For interassay imprecision, one positive and one low-positive sample are used. Each sample is tested one time on three different days. In case of a quantitative test or test system for detection of virus-specific antibodies or viral antigens, four positive and three low-positive samples are used for determination of intraassay imprecision, and two positive and one low-positive sample for determination of interassay imprecision. The corresponding recommendations for a quantitative NAT test or test system are three positive and three low-positive samples each for determination of intraassay imprecision and one positive and

Table 2 Verification of IVD/CE-Labeled and/or FDA-Approved Tests or Test Systems for Detection of Virus-Specific Antibodies, Viral Antigens, or Viral Nucleic Acid Testing

		No. of samples required			
		Detection of antibodies or antigens		Nucleic acid testing	
	Calibrator (sample)	Qualitative	Quantitative	Qualitative	Quantitative
Accuracy	Positive[a]	3	3	3	3
	Low positive[b]	3	3	3	3
	Negative	3	3	3	3
Intraassay imprecision	Positive[a]	1	4	1	3
	Low positive[b]	1	3	1	3
Interassay imprecision	Positive[a]	1	2	1	1
	Low positive[b]	1	1	1	1
Linearity	Positive[a]	0	1	0	1

[a]More than 1 $\log_{10}$ over the limit of detection (LOD) or the limit of quantification (LOQ) and within the upper limit of linearity of the test or test system.

[b]Up to 1 $\log_{10}$ over the LOD or the LOQ of the test or test system.

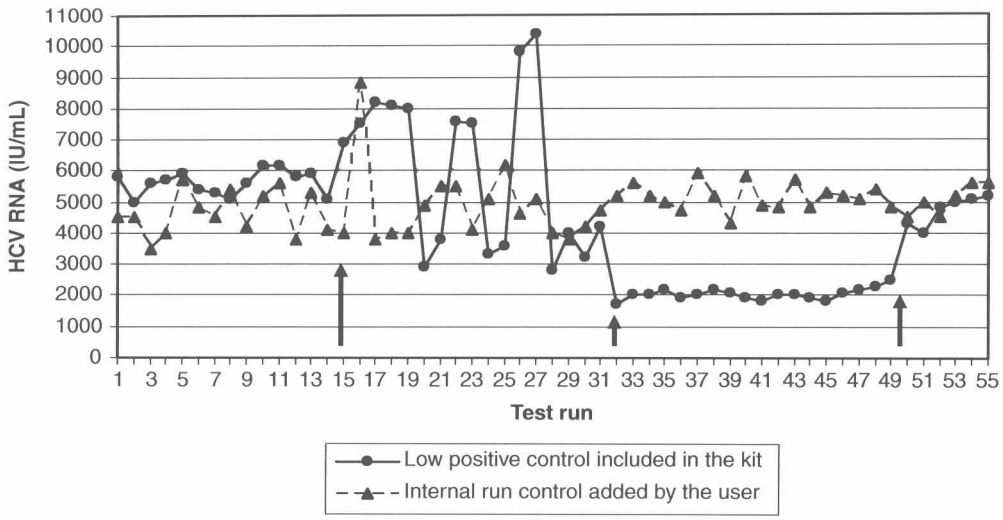


Figure 3 Performance of an internal run control (IRC) implemented in NAT (*arrows* indicating introduction of a new test lot).

low-positive sample each for determination of interassay imprecision. In order to optimize the verification workflow, it may be useful to take the first result of intraassay imprecision testing as first result of interassay imprecision testing thus allowing a reduction of the number of further runs for interassay imprecision testing to two. In case of a quantitative test or test system, linearity must be verified additionally by analyzing a serial dilution (tenfold dilution series with at least three dilution steps) of one positive sample in duplicate.

Additionally, it is recommended to survey the correctness of a test result obtained by an IVD/CE-labeled and/or an FDA-approved test or test system continuously after implementation in the routine diagnostic laboratory. This is achieved by introduction of an internal run control (IRC), which is independent from the positive control(s) included by the manufacturer of the test or test system and may be implemented either in each test run or within defined intervals. When introducing a new test lot, comparison of the results obtained by the IRC with those obtained by the positive control(s) included by the manufacturer of the test or test system enables identification of relevant aberrations at an early stage (Fig. 3). Statistical analysis of results obtained by both the IRC and the positive control(s) may also be helpful.

Minimum Requirements for Validation of a Laboratory-Developed Test or Test System for Detection of Virus-Specific Antibodies, Viral Antigens, or NAT

If a laboratory-developed test or test system for detection of virus-specific antibodies, viral antigens, or NAT is introduced in the routine diagnostic laboratory, validation experiments are performed to validate accuracy, recovery, selectivity, imprecision, and, in case of a quantitative test or test system, linearity (Table 3). For determination of the accuracy, three positive, three low-positive, and three negative samples are used. For recovery, 10 positive and 10 low-positive samples are tested. The selectivity of a test or test system for detection of virus-specific antibodies is determined by analyzing 10 negative samples including samples containing antibodies that may lead to cross-reactivity. For tests or test systems detecting viral antigens or NAT, 10 samples testing positive for antigens or viruses of the same family and samples spiked with reference material that may lead to cross-reactivity are analyzed. Each potentially cross-reactive analyte must be present in a high concentration (at least 10^5 $TCID_{50}$/mL or 10^5 genome equivalents/mL). Additionally, selectivity testing requires 10 low-positive samples including, for instance, samples with elevated hemoglobin levels, testing positive for rheumatoid factor, and/or containing auto-antibodies. Determination of intra- and interassay imprecision are similar to those for verification procedures except for an extension in the validation of quantitative tests or test systems regarding positive samples (use of six positives instead of three for

Table 3 Validation of a Laboratory-Developed Test or Test System for Detection of Virus-Specific Antibodies, Viral Antigens, or Viral Nucleic Acid Testing

		No. of samples required			
		Detection of antibodies or antigens		Nucleic acid testing	
	Calibrator (sample)	Qualitative	Quantitative	Qualitative	Quantitative
Accuracy	Positive[a]	3	3	3	3
	Low positive[b]	3	3	3	3
	Negative	3	3	3	3
Recovery	Positive[a]	10	10	10	10
	Low positive[b]	10	10	10	10
Selectivity	Negative[c]	10	10	10	10
	Low positive[b,d]	10	10	10	10
Intraassay imprecision	Positive[a]	1	6	1	6
	Low positive[b]	1	3	1	3
Interassay imprecision	Positive[a]	1	2	1	2
	Low positive[b]	1	1	1	1
Linearity	Positive[a,e]	0	2	0	2

[a]More than 1 $\log_{10}$ over the limit of detection (LOD) or the limit of quantification (LOQ) and within the upper limit of linearity of the test or test system.
[b]Up to 1 $\log_{10}$ over the LOD or the LOQ of the test or test system.
[c]Samples that may lead to cross-reactivity.
[d]Samples including possible interferents.
[e]Serial dilutions (at least four dilution steps) in duplicate on two different days.

determination of intraassay imprecision and two instead of one for determination of interassay imprecision). In the case of a quantitative laboratory-developed test or test system, linearity must be validated additionally by analyzing serial dilutions (at least four dilution steps) of two positive samples in duplicate on two different days.

Issues Regarding Introduction of a Laboratory-Developed NAT Assay

When establishing a laboratory-developed NAT assay, primer and probe sequences must be checked carefully by use of a genome sequence databank. It is advisable to verify the amplification product by means of sequencing and to use a primer pair that has already been published in a highly recognized journal. The latter helps to avoid testing of a more or less extended specificity panel. However, the published sequences should always be subjected to an alignment analysis by means of a genome sequence databank to ensure that the correct sequence has been published.

Moreover, several issues including the molecular technique employed, the detection format, introduction of an IC, and quantitation must be addressed. With regard to the molecular technique employed, it must be taken into consideration that automation reduces hands-on work and thus helps avoid human error. To ensure analyte-specific results, introduction of a probe detection format is required while melting curve analysis without probe detection format does not provide sufficient specificity. Because amplification may fail in a reaction due to interference from inhibitors, an IC must be incorporated in every NAT assay to exclude false-negative results. To ensure an accurate control of the entire NAT assay, the IC should be added to the sample before the start of the nucleic acid extraction procedure. Either a homologous or a heterologous IC can be employed. The homologous IC is a DNA sequence (for DNA amplification targets) or an in vitro transcript (for RNA targets) consisting of primer-binding regions identical to those of the target sequence, a randomized internal sequence with a length and base composition similar to those of the target sequence and a unique probe-binding region that differentiates