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Review

Quality assurance and quality control in the routine molecular diagnostic laboratory for infectious diseases

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Abstract

Molecular diagnostics has become one of the dominant platforms in clinical laboratory medicine. Technological improvements, from automated sample preparation to real time amplification technology, provide the possibility to develop and run assays for a growing number of clinical questions. However, quality assurance and quality control issues have often remained underdeveloped but are still critical. To relate patient results to prior results or to absolute values in clinical practice guidelines, those results need to be comparable across time and methods. This may be achieved either by producing the identical value across methods and test versions or by using reliable and stable conversions. The establishment of international standards and reference materials is thus of paramount importance. This review focuses on general and specific issues relevant for quality assurance and quality control in the routine molecular diagnostics laboratory.

Keywords: molecular diagnostics; quality assurance; quality control.

Accreditation issues

The framework of common standards including the international standards ISO 9001:2008 and ISO 15189 has been established allowing laboratories to plan and operate a medical testing with an effective quality management system that has strong elements of quality assurance, quality control, and quality improvement. When medical testing laboratories effectively implement this quality management system, they have continuous assurance that they are meeting their customers' needs and expectations for consistent, accurate, and timely test results.

Among several issues, these standards contain certain validation and verification procedures because the suitability of a laboratory technique does not necessarily prove that it would be performed correctly and provide valid results. To prove this, quality management systems have been implemented in the majority of routine diagnostic laboratories. For laboratories in the European Union, 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 (1). Additionally, common technical specifications enforced for tests or test systems are outlined in the Commission Decision of 7 May 2002 on common technical specifications for IVD medical devices (2). For laboratories in the US, the Food and Drug Administration (FDA) has established regulations based on current ISO standards (3). In addition to supervision, description, and conformity of processes by the use of standard operating procedures, the validation procedure focuses on the competence of the laboratory providing reliable test results and their correct interpretation.

Validation and verification work

As for all medical testing, laboratories performing molecular assays must adhere to established validation practices to ensure confidence and reliability in test results produced. It is worth noting, that the IVD Directive 98/79/EC includes not only the definition "test" but also the definition "test system" if more than a single component are required to generate a diagnostic result. For instance, molecular assays based on PCR usually consist of a combination of different reagents and instruments for nucleic acid extraction, amplification, and detection of amplification products. Only tests or test systems with proven suitability may be used in the routine molecular diagnostic laboratory, demanding verification work for each test or test system.

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Validation and verification work is defined as confirmation through the provision of objective evidence that requirements for a specific intended use or application have been fulfilled (ISO 9001:2008). Components of validation must be applied to ensure that a procedure, process, system, equipment, or method used works as expected and achieves the intended result constantly (WHO-BS/95. 1793). In contrast, components of verification are assigned to determine or confirm performance characteristics of a molecular test or test system before it is used for patient testing.

Components of validation

According to ISO 9001:2008 and ISO 15189, components of validation of a molecular test or test system include the implementation of an appropriate quality control regimen. This consists of both, internal and external quality controls for the new implemented molecular test or test system, the use of international standards and reference materials if available, validation of the employee competency, calibration procedures of the instruments used, and monitoring of the test results achieved in correlation with clinical findings regarding the diagnostic sensitivity and the diagnostic specificity of the molecular test or test system (Table 1).

Quality control

Because amplification may fail due to interference from inhibitors, an internal control (IC) must be incorporated in every molecular test or test system to exclude false-negative results. ICs are needed to exclude false-negative results due to interference from inhibitors. To ensure an accurate control of the entire molecular test system, 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 used. 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 the IC amplification product from the target amplification product (Figure 1). Either a single IC or multiple ICs for a set of molecular assays can be generated. In contrast to the homologous IC, the heterologous IC represents a second amplification system within the same reaction vessel (Figure 2). The control must have the same or similar extraction and amplification efficiencies as the target. Plasmids or housekeeping genes can be used as heterologous ICs. Any IC (homologous or heterologous)

Table 1 Components required for validation of molecular tests or test systems according to ISO 9000 and ISO 15189.

Component required	Particular requirements
Quality control: internal controls, external run controls, international standards and reference materials	Quality control records including performance data, instrument printouts, and corrective action
Proficiency testing participation for comparison of inter-laboratory test results	Proficiency testing results including corrective actions
Validation of employee competency	Key and operating staff qualifications, credentials verifying field expertise
Instrument maintenance and calibration	Instrument records including printouts of maintenance and calibration protocols
Correlation with clinical findings	Diagnostic sensitivity and specificity of the test or test system

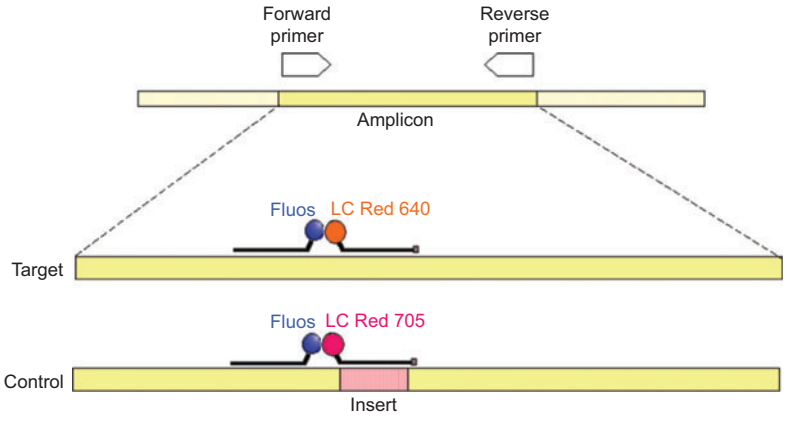

Figure 1 Homologous internal control showing an in vitro DNA transcript.
The primer annealing regions are identical to those of target sequence. One probe-binding region is identical to that of the target sequence, the second probe binding region is different to that of the target sequence (probe labeled with a different fluorescence dye).

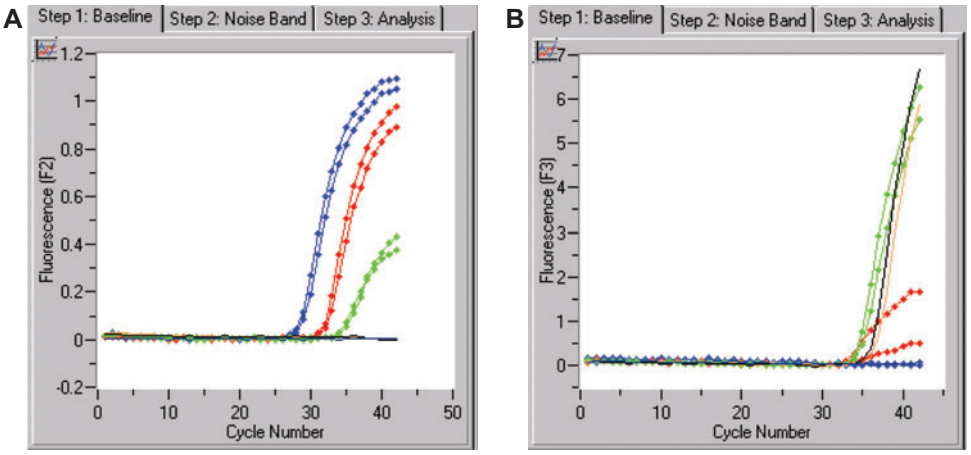

Figure 2 Real-time PCR including a heterologous internal control (human β microglobulin serving as a "housekeeping gene") by using a second amplification system in parallel in the same reaction vessel but with different fluorescence dyes.
(A) Target amplification curves (run in duplicate). (B) Internal control amplification curves. Note that the amplification of the internal control is competitively inhibited through that target with the highest concentration (blue curves).

must be added at a suitable concentration to prevent extreme competition with the target template for reagents.

To monitor the correctness of a test result obtained with the new molecular test or test system continuously after implementation in the routine diagnostic laboratory, the introduction of an external run control (ERC) which is independent from the external positive control(s) included by the manufacturer is recommended. Because the ERC must monitor the whole molecular assay including sample preparation, the ERC is added directly into the sample prior starting with nucleic acid extraction. An ERC may be implemented either in each test run or within defined intervals, e.g., when introducing a new test lot. Comparison of the results obtained by the ERC with those obtained by the external positive control(s) included by the manufacturer enables identification of relevant aberrations at an early stage (Figure 3).

An integral part of quality assurance is the use of well-characterized and readily available reference materials, to maintain clinical laboratory quality assurance for molecular tests or test systems testing. Reference materials can be used for quality control, verification of tests or test systems, detection of errors, monitoring of test performance, and proficiency testing. Without well-characterized and readily available reference materials, it is difficult to cross-reference molecular assays, IVD/CE labeled IVD/CE labeled or FDA-approved or -cleared molecular tests as well as laboratory-developed ones.

Recently, a hierarchy of reference materials has been described which is based on the degree of characterization of each material. The top category consists of *ISO Reference Materials*. These materials are sufficiently homogenous and stable with respect to one or more specified properties. They have been established for their intended use in a measurement process (4). *ISO Reference Materials* have been provided by organizations including the WHO as independent organization and several commercial distributors including the Institute of Reference Materials and Measurements (5). The second category consists of *ISO Certified Reference Materials*. These materials are characterized by a metrologically valid procedure for one or more specified properties, accompanied by a

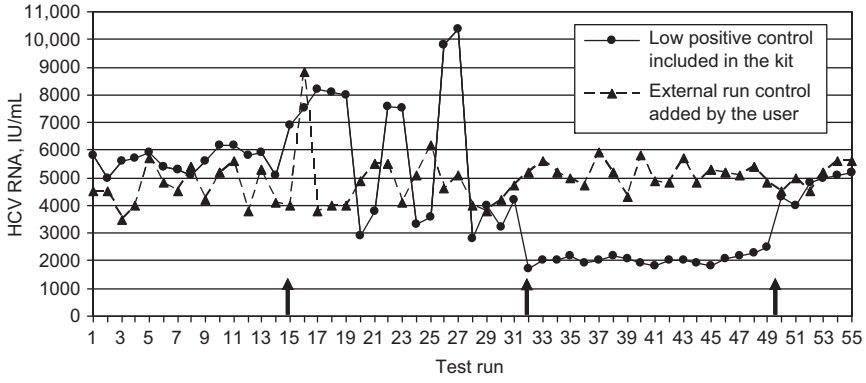

Figure 3 Performance of an external run control (ERC) implemented in a molecular assay for quantification of hepatitis C RNA (arrows indicate introduction of a new test lot).

certificate that provides the value of the specified property, its associated uncertainty, and a statement of metrological traceability (4). *ISO Certified Reference Materials* are provided, e.g., by the National Institute of Standards and Technology. They include commercially available reference materials, which either are CE-labeled (Conformité Européenne, a mandatory conformity mark for products placed within the European Union market) or approved or cleared for in vitro diagnostic use by the FDA.

For qualitative and quantitative molecular tests or test systems, metrologically valid procedures do exist. For Sanger sequencing, a possible approach to the development of a reference procedure was reported recently (6). However, for newly developed techniques including massively parallel sequencing, no metrologically valid procedure exists currently (7). In general, the number of reference materials developed by governmental organizations and manufacturers, which can be used for nucleic acid-based pathogen detection, is too small. This lack of widely available reference materials enforces the laboratories to use other types of materials as controls, such as the remainder of a patient specimen that was left following clinical routine testing. In addition, because reference materials are generally available in limited quantities, laboratories will need more widely available materials in the future that can be used on a daily basis for a variety of purposes, including quality control.

Proficiency testing

Proficiency testing is a process for checking actual laboratory testing performance, usually by means of inter-laboratory test results comparisons. Each medical testing laboratory must enroll in an approved proficiency program or programs, for each of the specialties and subspecialties for which it seeks accreditation. For example, to assess a laboratory's ability to use molecular diagnostic technologies within the clinical setting, taking part on proficiency testing panels providing the laboratory with a series of samples that resemble clinically significant specimens is mandatory. In Europe, several programs for proficiency testing in molecular diagnostics for infectious diseases have been established including *EQUAL-qual*, a project proposed under the auspices of the EC4 and funded by the European Commission (8), and several commercially available programs provided by, e.g., QCMD (9–13), NEQAS (14, 15), and INSTAND (16). Usually, a higher percentage of correct results is observed with IVD/CE labeled or FDA-approved or -cleared molecular tests or test systems than with laboratory-developed molecular assays.

Medical laboratories must be able to provide the proficiency testing results together with the individual reports to the national body of laboratory accreditation. Data must include test runs with results, reports, report lists, and signatures. A singular poor score in a certain proficiency-testing program does not mean that the test or test system must be abandoned immediately. However, immediate action must be taken to overcome the existing deviation(s). The unsuccessful correction of the existing deviation(s) can lead to suspension of the test or test system affected by the national body of laboratory accreditation.

Validation of employee competency

The laboratory staff must remain competent in performing molecular tests and test systems and in reporting valid results. A large number of methods are available for the acquisition of the specific knowledge. There are a number of providers offering trainings on molecular diagnostics. Both key and operating staff must participate and complete trainings successfully, yielding credentials that verify field expertise on a constant level. These procedures eventually help to ensure the consistency of the produced and reported results by the molecular diagnostics laboratory.

Instrument maintenance and calibration

Instrument calibration, instrument maintenance, and function checks contribute to the on-site assessment of a medical testing laboratory. To comply with relevant standards, a documented and recorded program of maintenance and calibration following the manufacturer's recommendations is required. Additionally, the manufacturer's instructions, the operator's manuals, and updates of documentation must be used to meet defined criteria (e.g., frequency and modification of procedures) regarding maintenance and calibration.

Correlation with clinical findings

The implications of a false-negative or a false-positive test result and the impact of a laboratory result on the diagnosis and management of the disease require consideration regarding the diagnostic sensitivity and the diagnostic specificity of a test or test system. The diagnostic sensitivity of a test or test system is determined by the proportion of patients with well-defined clinical disorders whose test values are positive or exceed a defined limit of decision (i.e., a positive test result and identification of the patients who have a disease). It must be considered carefully that the clinical disorder must be defined by criteria independent of the test or test system used. The term *diagnostic sensitivity*, which is mainly used in the EU, is equivalent to *clinical sensitivity* mainly used in the US. The diagnostic specificity of a test or test system is defined by the ability of a measurement procedure to measure solely the measurand, thus avoiding false-positive test results (i.e., a negative test result and identification of the patients where the disease is absent). The term *diagnostic specificity* is used uniformly in the EU and in the US.

Components of verification work

Following successful implementation of the components of validation, verification of molecular tests or test systems

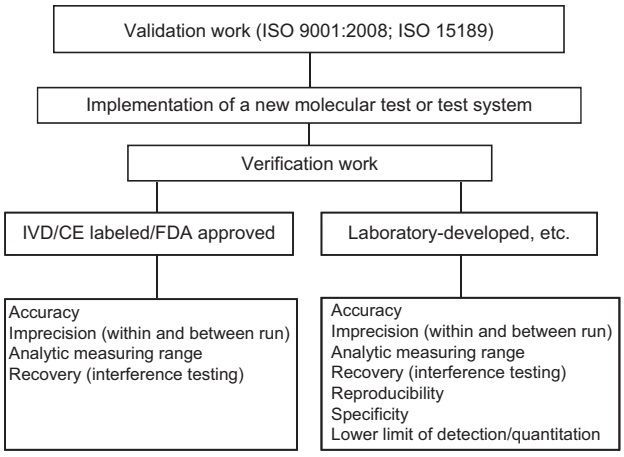

Figure 4 Workflow for implementing a new molecular test or test system in the routine diagnostic laboratory.

must be performed. Because clear and comprehensive guidelines on verification of molecular tests or test systems are lacking, a great diversity of approaches exists. This diversity is not only seen within the EU in general, but also often even within certain countries. Therefore, a common national or preferably an international regulatory framework on verification of molecular tests or test systems is urgently needed. Suggestions described here may be a helpful approach towards a clear and meaningful verification procedure when implementing a new molecular test or test system in the routine diagnostic laboratory (Figure 4).

Components of verification of a molecular test or test system should depend on the type of assay used in the laboratory. For IVD/CE labeled and FDA-approved or -cleared tests or test systems, the manufacturer is responsible that the IVD achieves the performance as stated. However, it is advisable to determine or confirm performance characteristics before used for patient testing. In contrast, for laboratory-developed tests or test systems, research-use-only (RUO) tests or test systems, or the combination of different IVD/CE labeled or that of different FDA-approved or -cleared tests without recommendation of the manufacturer, or any change of a compulsory test procedure, the laboratory is responsible for both the suitability and the correct performance of the test or test system demanding for an extended verification procedure before it is used for patient testing.

Components of verification work for IVD/CE labeled and FDA-approved or -cleared tests or test systems

This includes testing of accuracy, within run and between run imprecision, and determination of the analytic measuring range (linearity). If a sample matrix is intended being introduced for which the manufacturer has not verified the assay, recovery testing to rule out possible interfering substances should be performed additionally (Table 2).

Table 2 Components required for verification of IVD/CE-labeled and/or FDA-cleared tests or test systems.

Component required	Assigned value of the accepted reference material
Accuracy	Positive[a]
	Low positive[b]
	Negative
Imprecision (within and between run)	Positive[a]
	Low positive[b]
Analytic measuring range[c]	Positive[a]
Recovery (interference testing)	Positive[a]

[a]More than 1 $\log_{10}$ over the LOD and within the upper limit of linearity of the appropriate reference material. [b]Up to 1 $\log_{10}$ over the LOD of the appropriate reference material. [c]In case of a quantitative test or test system.

Components of verification work for laboratory-developed tests or test systems

To determine or confirm performance characteristics of a laboratory-developed test or test system, RUO test or test system, or the combination of different IVD/CE labeled tests or that of different FDA-approved or -cleared tests without recommendation of the manufacturer before it is used for patient testing, extended verification including additional components of verification must be performed. The additional components include testing of reproducibility, testing of specificity, determination of the limit of detection (LOD) in case of a qualitative molecular test or test system, and determination of the limit of quantitation (LOQ) in case of a quantitative molecular test or test system. In any case, for accurate reporting of verification studies, objective and statistically valid methods need to be employed (17).

Reporting results

Treatment of a patient mainly depends on the report obtained from the laboratory. The result should be reported clearly and briefly. Modern developments have shown an increased interest in utilizing measurements for the detection of trace levels of DNA. Such low-level detection methods are central to regulatory, public health, medical, and quality control issues. There is little in the way of standardization of data handling from these methods, and the data generated need to be analyzed appropriately if the results are to be interpreted correctly. For many molecular tests, results need to be interpreted.

Qualitative assays mostly have clear results, which could be “detectable” (or “positive”) or “not detectable” (or “<LOD”). It is of major importance to add always the LOD of the assay used. In some cases, further explanation is required for the interpretation of the result obtained and its clinical relevance.

There are three categories of results when using quantitative real time PCR (Figure 5) (1). If the value obtained is above the upper limit of quantitation, the value is reported as “>analytic measuring range” (2). If the value obtained is

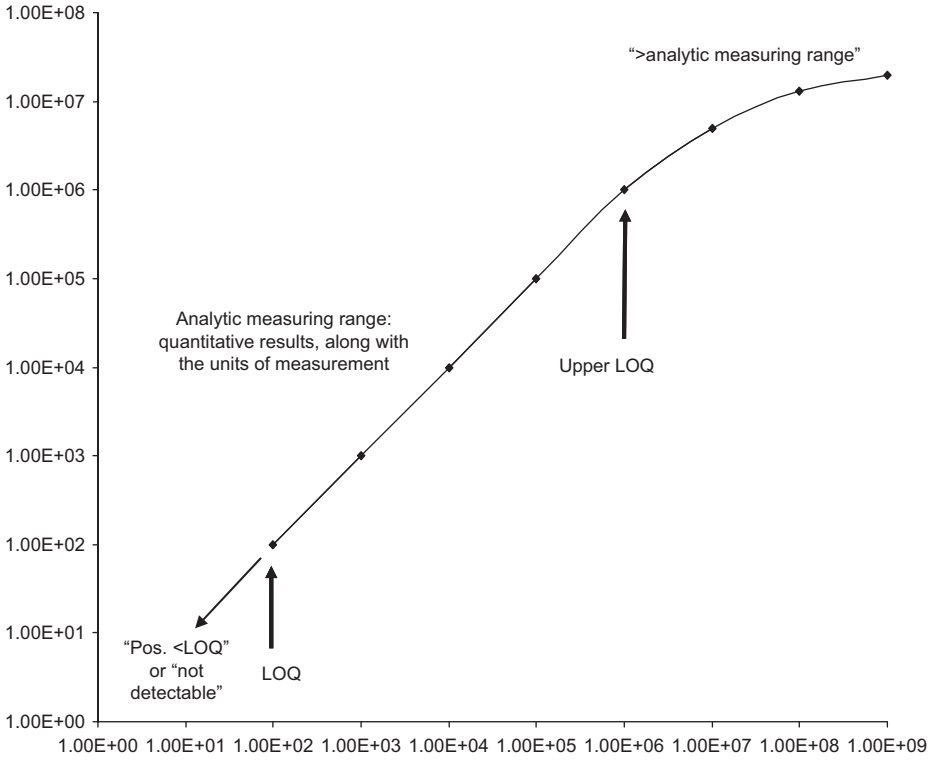

Figure 5 Categories of results obtained with a quantitative real-time PCR assay.

within the analytic measuring range, the value is reported quantitatively, along with the units of measurement (3). If the value obtained is under the LOQ, the result reported may be detectable but not accurately quantifiable ("positive <LOQ") or "not detectable". It is of major importance to state always the analytic measuring range of the assay employed.

The reproducibility of a molecular test or test system is another important parameter, which helps the physician to determine whether two consecutive results are considerably different from each other. Information about reproducibility of the assay can be sent along with the report or it should be easily available to the physician. Furthermore, it is useful to report whether a detected microbe is interpreted as pathogen or part of the normal flora. False-positives or false-negatives are the main disadvantage of most of the laboratory results. It would be important to address these limitations in the report depending on the nature of each assay and the significance of an incorrect result.

Conclusions

For validation work, harmonization among the framework of common standards (ISO 9001:2008 and ISO 15189) does exist. In contrast, no harmonization exists for verification work currently; however, verification work is mandatory if any new molecular test or test system for patient testing is introduced. Verification work helps to ensure reliable test results and contributes to a better comparability of molecular tests and test systems. Prior to the use for patient testing, all molecular tests and test systems need to be verified. Laboratory-developed tests or test systems, RUO tests or test systems, and the combination of different IVD/CE labeled and/or FDA-approved or -cleared molecular tests or test systems without recommendation of the manufacturer are subject to an extended verification procedure. When reporting results, well-defined terms need to be used including "detectable" (or "positive") or "not detectable" for a qualitative assay and ">analytic measuring range", any quantitative value, "positive <LOQ", or "not detectable" for a quantitative assay.

Conflict of interest statement

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