Original Article

REFLECTIONS AND PROPOSALS TO ASSURE QUALITY IN MOLECULAR DIAGNOSTICS

Raymaekers M[1], Bakkus M[2], Boone E[3], de Rijke B[4], El Housni H[5], Descheemaeker P[6], De Schouwer P[7], Franke S[8], Hillen F[1], Nollet F[6], Soetens O[9], Vankeerberghen A[10], on behalf of the MolecularDiagnostics.be working group

[1]Laboratorium voor Moleculaire Biologie, Klinisch Laboratorium, Jessa Ziekenhuis, campus Virga Jesse, Hasselt, België, [2]Laboratorium voor Moleculaire Hematologie, UZ Brussel, Brussel, België, [3]Laboratorium-Moleculaire Diagnostiek, H.-Hartziekenhuis Roeselare-Menen, Roeselare, België, [4]Laboratorium Klinische Biologie, AZ St-Lucas-Volkskliniek, Gent, België, [5]ULB-Erasme, Service de génétique médicale, Hôpital Erasme, Bruxelles, Belgique, [6]Laboratorium Klinische Biologie, AZ Sint-Jan Brugge-Oostende AV, Brugge, België, [7]Klinisch Laboratorium ZNA, Ziekenhuis Netwerk Antwerpen, Site Stuivenberg, Antwerpen, België, [8]Laboratoire de Biologie Moléculaire Hématologique, Centre de Génétique Humaine CHU Liège , Domaine Universitaire du Sart Tilman, Liège, Belgique, [9]Laboratorium Microbiologie en Ziekenhuishygiëne, UZ Brussel, Brussel, België and [10]Laboratorium Moleculaire Biologie, OLVZ-Aalst, Aalst, België

Correspondence and offprint requests to: Marijke Raymaekers, E-mail: marijke.raymaekers@jessazh.be

ABSTRACT

Molecular diagnostic testing has become an important tool in clinical laboratories. Accreditation according to the international quality standard ISO15189:2007 for medical laboratories is required for reimbursement of several molecular diagnostic tests in Belgium. Since the ISO15189:2007 standard applies to medical laboratories in general, the particular requirements for quality and competence are mentioned in general terms, not taking into account the specificities of molecular biology testing. Therefore, the working group "MolecularDiagnostics.be" described a consensus interpretation of chapter 5, Technical requirements, of the ISO standard for application in molecular diagnostic laboratories. The manuscript can be used as an instrument to prepare internal and external audits that meet the ISO15189:2007 (chapter 5) criteria.

Key words: molecular diagnostics, quality assurance, ISO15189:2007, PCR

INTRODUCTION

Since the first description of the polymerase chain reaction (PCR) by Saki et al (1), nucleic acid (NA) testing has become an even more important tool in research and clinical diagnostics. These molecular diagnostic tests are technically demanding, expensive and need a high degree of quality assurance. From 2001 up to now, several proposals for reimbursement of molecular diagnostic tests in Belgium were elaborated, which, at present, have resulted in reimbursement of a limited number of tests for which indications have been described in the Royal Decree of June 7th 2007 (art 33bis) and in the Royal Decree of March 19th 2008 (art 24bis). However, reimbursement is granted only to those laboratories that are accredited according to the ISO-standard (ISO15189:2007) (2). At this moment only few laboratories obtained accreditation for molecular diagnostic tests, many others are in the process of applying for accreditation.

Since many aspects of the standard are topic of debate, there is a need for consensus interpretation of the different aspects of the standard among Belgian laboratories. Moreover, the ISO15189:2007 is prone to auditor dependent interpretation differences, and does not contain any suggestions concerning the implementation of the standard in daily practice. A group of Belgian molecular biologists and clinical biologists, joined together in the working party "MolecularDiagnostics.be", discussed the elements described in chapter 5 of the ISO 15189:2007 standard. The main goal was to reach a consensus regarding the interpretation of chapter 5 of the standard for molecular diagnostics laboratories.

METHODS

The Belgian working group (MolecularDiagnostics.be), consisting of molecular biologists and clinical biologists (39 members in January 2009), affiliated to hospital laboratories and active in the field of molecular diagnostics, was founded and a website (http://www.MolecularDiagnostics.be/) was created as a forum for collaboration. The first goal of the working group was to collaborate on the standardization of molecular diagnostic testing as described by chapter 5 of the ISO15189:2007. The objective was to propose a general consensus interpretation regarding suggestions on how to fulfil the criteria of the ISO15189:2007 standard. Eight subgroups of minimal 4 persons, containing at least one person from the microbiology field and one from the haematology field, were formed, each tackling a topic of chapter 5. For topic 5.4 (storage conditions) and 5.5 (amount of samples used for the validation), an enquiry was sent to all 39 members of the working group. The consensus text of each subgroup, proposing an approach to the ISO criteria was discussed in two subsequent general meetings and after agreement of all members a final text was constructed. When no consensus could be reached, this is mentioned in the text.

For these recommendations, the focus was on nucleic acid amplification as this is the most frequently used technique in molecular laboratory testing. For *HER2/NEU* testing by FISH, Belgian guidelines were already published in 2007 (3).

RESULTS AND DISCUSSION

1. Personnel (§5.1)

Although molecular diagnostic assays are routinely used in a lot of clinical laboratories, molecular diagnostics is still believed to be a specialized discipline requiring personnel that is specifically and adequately trained in the field of molecular biology based diagnostic techniques.

We propose a generalized structure for a molecular diagnostics laboratory with the following functions:

Routine molecular diagnostic testing should be performed by medical laboratory technician(s). They should have an adequate education and be trained according to their job description, with special attention to the molecular diagnostic techniques which are performed in the laboratory. Besides the required technical skills, knowledge of the basic theoretical principles of molecular biology, especially concerning contamination prevention, is necessary.

Technical supervision is preferentially performed by a molecular biologist(s) or scientific collaborator(s). In practice, this function is fulfilled by a scientific collaborator, a medical laboratory technician or a medically trained person depending on the organisation of the laboratory. We do not wish to make a statement on how a laboratory should fulfil this function but we strongly advice that technical supervisors should be thoroughly trained in molecular biology. This can be in part guaranteed by an academic degree, preferentially in the field of molecular biology, but practical experience is nevertheless recommended. In this respect, it is advised that the technical supervisor keeps up-to-date with peer reviewed literature and attends relevant meetings and symposia on a regular basis.

The technical supervisor is in most laboratories involved in the technical validation of test results, technical problem solving, the development and validation of home-brew and/or FDA/CE-IVD labelled assays, the validation of equipment, education and training of the medical laboratory technicians, organization and monitoring of the quality system and preferentially has an advisory role in the molecular biology laboratory policy. Depending on the organization of the laboratory, the technical supervisor can also be responsible for the organization of the daily laboratory work.

Medical supervision should be fulfilled according to Belgian INAMI-RIZIV legislation.

2. Accommodation and environmental conditions (§5.2)

One of the disadvantages of NA amplification techniques (e.g. PCR, NASBA and SDA) that are commonly used in molecular diagnostic testing, is the fact that they are prone to contamination, which can lead to false positive results. To reduce the possibility of false positive results due to contamination, infrastructure and environmental conditions have to be designed with the focus on contamination prevention.

Working in at least two separate rooms is mandatory: a pre-PCR room and a post-PCR room. In the pre-PCR room, all procedures which do not generate amplicons are allowed. Handling of PCR products may never take place in the pre-PCR room. Furthermore, dedicated working areas for the preparation of the PCR-mixture and the extraction of NA are preferred. These working areas may be equipped with a dead air box (PCR-mix) and/or a biohazard safety cabinet for sample preparation and NA extraction. The amplification reaction and detection of amplicons should take place in a post-PCR room or in a dedicated part of the post PCR-room. Furthermore, we advise to minimize the traffic from post-PCR to pre-PCR by handling an adequate work-flow (4) and usage of equipment, material and storage facility should be dedicated to each separate room and labelled in a clearly visible way.

No consensus was reached about the use of over- and under-pressure in the pre and post-PCR rooms as a measure for contamination control. However, it was agreed that if this measure has been taken, the difference in pressure should be monitored.

Besides infrastructural measures, several procedures for eliminating potential contamination sources can be followed (5-11). In most molecular diagnostic laboratories these contamination prevention strategies, known as good laboratory practice (GLP), have been introduced. According to these references, these procedures include physical separation of laboratory sections and cleaning of working surfaces and instruments with hypochlorite or a NA decontamination solution. The use of hypochlorite as decontamination solution has been described in different concentrations and incubation times and is described as being amplicon length dependent (10, 12).

Also use of aliquoted solutions, disposable gloves, positive displacement pipettes with disposable tips and "premix" reagents is recommended (11). In addition, Uracyl-N-Glycosylase (UNG)-chemistry, photochemical inactivation, treatment with DNAse I or exonuclease III and UV-irradiation can be adequate contamination prevention measures. UV-irradiation should be seen as an additional precaution rather than as a

replacement for careful laboratory practice (10). The efficiency of irradiation with UV light is dependant on several parameters (the distance to the surface, the intensity of the UV light, the wavelength of the UV light and irradiation time) and must be determined empirically. Dry DNA has been described to be inactivated only slowly by UV-irradiation (13).

Nevertheless, it is advised to set up validated procedures for contamination prevention and monitoring.

Apart from the measures taken for contamination prevention, there should be adequate procedures and controls available to monitor possible contamination (11). These contamination controls include the use and follow-up of negative extraction and amplification controls (see below paragraph 5.6). In case of contamination, the use of swab experiments of the working areas, pipettes, clothing, freezers, fridges, telephones, door handles and instruments can help in finding the source of contamination.

In the near future, routine molecular diagnostic testing will further evolve with the introduction of automation. These automates do not fit in the pre- and post-PCR laboratory setting since they usually combine extraction, reaction setup, amplification and detection in one platform. Especially for these automates, adequate controls and measures for contamination prevention and contamination detection should be implemented.

3. Laboratory equipment (§5.3)

Several instruments such as thermal cyclers, pipettes, refrigerators and freezers are considered critical in the molecular diagnostic laboratory.

Many brands and types of thermal cyclers are currently on the market. Each cycler has its own technical specifications and performance characteristics (e.g. accuracy at a certain temperature, over- and undershoot of the temperature profile, temperature uniformity). Therefore, assays should be validated on each type of cycler on which they will be performed (14).

Possible malfunctioning of thermal cyclers can be monitored in two ways. Firstly, proper sensitivity controls should be used in each experiment to detect abrupt mal-functioning of an instrument (11). Secondly, a regular dynamic temperature control (preferentially at least once a year) is encouraged to detect temperature drifts of the instrument (e.g. Cyclertest). This temperature profiling can be replaced by a highly sensitive test, for instance based on melting curve analysis, which verifies the correct temperatures set on the instrument (e.g. OTV rotor,…).

It was the experience of several labs that manufacturers' specifications are set too stringent. As a consequence many cyclers do fall outside the manufacturers' own specifications after a couple of years. In this case we propose that instruments can still be used if special precautions are taken. These could be 1) determination of the coldest and warmest spot of the cycler and positioning of sensitivity controls on these locations or 2) validation of the PCR reaction performance at the temperature range the instrument is reaching at that moment. In any case, it should be clearly described when the instrument needs to be taken out of use. Finally, after repair and service, the instrument should be checked. For this purpose a routine test can be used only on condition that adequate controls are included. A repair and service agreement between the laboratory and the service company should clarify the role of both parties. The service engineer has to guarantee that the instrument is returned in an optimal condition to the laboratory. A maintenance scheme should be present and traceable for all instruments, including precision instruments like pipettes.

Volumetric control of pipettes should be performed with a frequency according to the frequency of use and importance of accuracy of the pipettes, but at least once a year. The performance specifications are dependent on its usage (critical pipettes versus non-critical). It was debated that manufacturer's specifications are in general very stringent to reach. Therefore some labs have doubled the manufacturer's specifications and use their own specifications. No consensus could be reached regarding this matter.

When handling pipettes for maintenance and calibration, special care should be taken to prevent contamination. In this respect, it is advised to decontaminate post-PCR pipettes before transport and to separate them from pre-PCR pipettes (e.g. use separate bags for transport).

Most samples and reagents that are used in molecular diagnostic testing need refrigeration or freezing for storage. Medical grade refrigerators/freezers seem to be preferred although recently a manuscript by Cray et al seems to suggest equal performances for medical grade and non-medical grade refrigerators/freezers (15). In our opinion, non-medical refrigerators/freezers can also be used, if properly working and controlled. Entry validation and temperature monitoring are necessary for freezers and refrigerators and adequate non-conformity procedures should be available.

We believe that correct procedures for reagent use are important for assuring quality. Most reagents (dNTPs, primers, probes, enzymes, etc.) are particularly sensitive to freeze/thawing. It is important to keep track of the number of freeze/thawing cycles for these products or to minimize the freeze/thawing cycles by making aliquots. Most companies who provide primers and probes do not mention expiry dates. It is our experience that, if properly stored, primers are highly stable and can be stored for up to 5 years. Probes may be less stable, depending on the type of fluorescent and quencher groups incorporated.

It is strongly advised to perform an entry control of critical reagents especially primers and probes. Synthesis of custom made primers and probes may vary according to variations in the chemical synthesis performance. The entry control can be done by parallel comparison of the new reagent with the reagent currently in use. These comparisons should be documented. Not all companies perform a quality control on all primers and probes they manufacture. At present, some companies offer medical grade oligonucleotides, but these are very expensive and must be bought at a high quantity. We believe that use of these reagents is not mandatory if an adequate entry control is performed.

Reference and control materials are also preferentially tested before use, especially when these consist of RNA. The entry control can be done by parallel comparison, which should be documented, of the new reagent with the material that is currently in use. For quantitative testing this comparison should be quantitatively interpreted. It is necessary to have a certificate, stating the contents and the quantity of the reference material that is used.

For other reagents, entry control is not considered mandatory if they can be re-ordered and tests can be repeated within an acceptable answering period. On the other hand, for urgent tests, entry validation of the reagents seems to be advantageous. Alternatively, a backup procedure could be that samples are sent to another laboratory.

4. Pre-examination procedures (§5.4)

As for all clinical laboratory testing, clear and completely filled out analysis request forms are required. It is also obvious that patient samples should be traceable and clearly identified. For particular tests, e.g. Herpes simplex virus detection, enterovirus detection and BCR-ABL (INAMI-RIZIV nomenclature art 24bis and 33bis), clinical information is required for reimbursement in Belgium. This information also enables correct medical validation and interpretation of the results. When the requested information remains unavailable, it is advised to give a notice of this on the test report.

Molecular diagnostic tests are generally performed on DNA or RNA extracted from a clinical sample. RNA is very sensitive to degradation which might negatively influence the result (e.g. detection of minimal residual disease or low viral loads). Therefore, the laboratory should provide clear instructions, preferably in a laboratory handbook available on the internet, concerning sample type, sampling conditions, sample volume, transportation conditions, and storage conditions and time of primary samples and NA. A separate sample aliquot for molecular testing is preferred both for the above mentioned storage and transport conditions and for the prevention of possible sample or amplicon cross-contamination. Any non-conformity to the sampling procedure should be noted as a comment on the result report.

Up to now, only few small scale studies on the stability and storage conditions of primary samples have been published and only limited guidelines are available (16-21). A guideline published by the Clinical Laboratory and Standards Institute (22) describes procedures for the collection, transport, preparation and storage of specimens for molecular methods.

However, for each sample type, the sample collection, storage, and stability features should be validated by peer-reviewed reference articles, by recommendations from the FDA/CE-IVD assay manufacturers or by validation performed in the lab. After enquiry of the participating laboratories, it was clear that only few labs have validated transport and storage conditions of primary samples and purified NA. The enquiry showed that most of the primary samples are transported to the laboratory at ambient temperature, even for RNA targets. Storage conditions of the primary sample after arrival in the lab varied depending on the specimen type, analyte (DNA or RNA) and/or organism being tested but also on the laboratory. Some laboratories have experienced that DNA can be stored a long time at 4°C without loss of quality, while others store the DNA at –20°C. As generally accepted, long-term storage of purified cDNA is best at temperatures below - 20°C and purified RNA at a temperature below - 70°C, although few labs have validated this. It was generally accepted that freeze-thaw cycles of RNA should be avoided and aliquoting is recommended.

In the near future this working group shall focus on transport conditions, on sample preservation conditions before NA extraction, and on NA storage conditions.

5. Examination procedures (§5.5)

The guidelines described by Evidence Based Laboratory Medicine (EBLM) (23), and the critically appraised topic (CAT) (24) are valuable tools in the selection of an examination procedure. These guidelines can be applied to molecular diagnostic tests. It is generally accepted that at least the following aspects should be taken into account, discussed and documented whenever a new diagnostic test is selected for introduction in the laboratory (Fig. 1).

1. Scientific literature evidence found in peer reviewed articles, guidelines, expert opinions.
2. The patient population for which the test will be designed has to be well defined since it might influence the other test selection criteria.
3. The selection of an adequate sample type has to take into account the ease of sample collection, the minimal sample volume needed, the recipient type (type of container, additives), the transport conditions in respect to the stability of the material, and the available literature evidence (peer review articles, guidelines).
4. The technique used for the examination procedure should be compatible with the required turn around time.
5. Practical consequences of the implementation of a new examination procedure in the current laboratory setting should be considered.
6. Technical and diagnostic test performances should meet the clinical needs. It is obvious that the opinion of the clinician requesting the examination procedure is of high value when the introduction of a new test is considered.
7. The total cost of the test should be calculated and should be in balance with the clinical and financial impact of the test result.

Figure 1: *Issues to be considered when a new diagnostic test is selected.*

Before the collection of validation data starts, the postulated aims should be formulated. This relates to the selection criteria mentioned above. During the validation process, the clinical and technical performances of the test have to be documented. The blueprint described in table 1 is based on the blueprint described by Raymaekers et al (25) and can be used as a template to guide this process. The exact interpretation is depending on the platform or assay under validation. Some of the items might not be applicable and are preferentially recorded in the validation report as “not applicable” (together with some explanation on the reason why) indicating that these items were taken into account. The blueprint may either be used:

(a) For implementation validation of FDA/CE-IVD labelled assays/methods if the procedure is followed completely as described (in this document referred to as: “FDA/CE-IVD”)

(b) For implementation validation of assays/methods from peer reviewed multicentre (at least three centres) publications if the procedure is followed completely as described (in this document also referred to as: “FDA/CE-IVD”)

(c) For the extended validation of home-brew assays, research-use only assays, and assays published in peer reviewed publications or FDA/CE-IVD labelled assays that are modified by the user (in this document referred to as: “home-brew”).

Table 1: BLUEPRINT

I. Aims:
  1. Clinical purpose (patient population, sample type)
  2. Assay and target
  3. Expected performance (technical, clinical)

II. Validation:
  1. Choice of method (theoretical)
     i. Sample type
     ii. Examination procedure
     iii. Literature study
     iv. Extraction/sample preparation method
     v. Target gene (is also applicable for FDA/CE-IVD labelled assays) (nucleic acid target, chromosomes,…), oligonucleotide sequences, chromosomes
     vi. Blast search
     vii. Method of detection
  2. Technical validation
     A. Optimization:
        - Optimization shall be performed for the entire procedure, starting from sample collection to reporting of result.
        - The method of analysis should be optimized such that the postulated technical performance is obtained. Parameters can include $MgCl_2$, primers, probes, sample collection, extraction conditions, PCR conditions.
     B. Performance characteristics: (cfr table 2)
        - The validation is matrix dependant [26, 30]. Performance characteristics should be available for each matrix that shall be used. The matrix may also include DNA/RNA.
        - Standard material (EQC, WHO standard) is preferentially used for determining the performance characteristics. If standard material is not available, the following material can be used if it is well documented by a reference technique: a commercially available control, patient material, spiked material, plasmids. When there is no quantified reference material available, a relative quantification can be obtained by the use of EQC samples.

        i. Precision (Inter and intra run)
           Definition: the precision is defined as the level of concordance of the individual test results within a single run (intra-assay precision) and from one run to another (inter-assay precision) [26].
           Characterized by: standard deviation of the measurements and coefficient of correlation.
        ii. Trueness (accuracy)
           Definition: Trueness 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.[26]
           Characterized by: percentage agreement with the reference method/material [28].
        iii. Linearity (measuring range)
           Definition: the linearity is defined as the determination of the linear range of quantification.
           Characterized by: regression coefficient (ideally 1) after linear regression.
        iv. Limit of detection (LOD)/analytical sensitivity
           Definition: the LOD is the lowest concentration or quantity of an analyte where ≥ 95 % of test runs give positive results, following serial dilutions of an international reference material, calibrated reference material or sample, tested under routine laboratory conditions (COI = 95 %).[28-30]
        v. Limit of quantification (LOQ)
           Definition: the LOQ is the lowest and highest concentration of analyte that can be detected with acceptable precision and accuracy, under routine laboratory conditions. These concentrations establish the measuring range for the assay.[30]
        vi. Analytical specificity
           Definition: the analytical specificity is defined as the method's ability to obtain negative results in concordance with negative results obtained by the reference method.[30]
     C. Interfering substances:
        Measures should be taken to detect the presence of interfering substances and to assess the quality of the extraction (detection of efficient extraction, carry over)
  3. Clinical validation
     Results of molecular diagnostic test should always be correlated with other results and with the clinical context.
     The assay can be used in routine diagnostic setting after a minimal clinical validation and evaluated more extensively afterwards.
     Clinical validation can include the following:
     i. Clinical performance
     ii. Correlation to disease or disorder
        a. Negative predictive value
        b. Positive predictive value
     iii. Comparison to current methods

III. Conclusion

For some parameters, reference material is commercially available for the validation of assays. Most valuable are proficiency panels (samples provided by QCMD, Instand, SKML, UKNEQAS), DNA/RNA panels from commercial companies (e.g. Acrometrix, Ipsogen, Invivoscribe) and cell lines and standards from the NIBSC. Of course, these can only be used as reference material if a clear statement concerning content and/or concentration is provided.

Unfortunately, no reference material is available for all parameters and sometimes the sample matrix is different compared to clinical samples. In these cases, clinical samples characterized by a second method can be used for validation. This second method (possibly performed in another laboratory) can be a previous or alternative (validated) method (e.g. sequencing, correlation to disease or disorder). In case of discordant results a third method has to be used. The samples can be either patient samples or spiked samples (with plasmids, reference material). Patient samples are preferred over spiked samples but spiked samples can be used if positive samples are scarce. If there is no

reference material available, both the determination of the limit of detection and absolute quantification are not possible (26) and a relative statement on the target load can be used (26, 27).

For quantitative assays, plasmids used for standard curves should ideally be calibrated to an international unit or international standard. However, at this moment those exist only for a few parameters (e.g. HCV). In that case, correlation can be performed by use of EQC panels.

It should also be documented and validated which methods, formulas, calculations are used to transform raw data into a quantitative reportable result (for instance in international units or other standard), including the software used for these calculations.

Finally, a review of procedures must be undertaken and documented at a defined interval and can include a study of recent literature, analysis of complaints concerning the procedure, evaluation of quality controls, a comparison of the frequency of the observed positive and negative results with literature data, and/or by review of the data together with data from other complementary assays, if available.

There was no consensus on the amount of samples that should be used for the determination of performance characteristics and the clinical validation. It was agreed that the sample number should be statistically relevant but it is clear that for some assays the amount of positive samples is scarce. Also the high cost of a molecular diagnostic test is an obstacle for a large validation. Although we propose a certain amount of samples in table 2, based on literature (26, 28-30), a questionnaire revealed that only few laboratories test a large amount of samples. In the near future this working group will focus on standardization of routine assays performed in multiple labs.

6. ASSURING QUALITY OF EXAMINATION PROCEDURES (§5.6)

To ensure quality of molecular diagnostic tests, it is essential to include adequate controls in each analysis. At a second level, quality of test results should be monitored on a regular basis by participation in an external quality control scheme, or by inter-laboratory comparison.

In each experiment it is mandatory to include a negative amplification control (no template control; NTC) to monitor contamination of reagents. However, it is advised to use a negative extraction control with each sample preparation/ extraction experiment to monitor possible carry-over contamination throughout the whole procedure, including the sample preparation and extraction phase. This is mostly done by replacing the primary sample by water. Moreover, if inhibition is an important issue, an internal control may be included for each sample to detect false negative results.

As a general rule, a positive amplification control should also be included in each experiment. The positive amplification control may be a positive patient sample, spiked sample or NA (DNA, RNA and cDNA). The use of a sensitivity control, which results in a reproducibly positive detection just above the level of sensitivity of the assay, is recommended for assays where sensitivity is of importance. There was no consensus whether this sensitivity control is sufficient for quantitative assays. Based on the CLSI MM3 guidelines, it is preferable to combine the sensitivity control with a high positive control (31). For quantitative assays the positive control must be quantified, and the result of this quantification needs to be within a pre-defined range. For real time PCR results there was no consensus whether the interpretation should be done on Ct values or on the quantitative value.

Table 2: Performance characteristics.

	FDA/CE-IVD Peer review multicentre publications	Home brew Adapted FDA/CE-IVD Adapted peer review multicentre publications
Precision (inter and intra run)	1 low positive sample, 1 high positive sample 3 replicates within 3 days. Preferentially from extraction.	1 low positive sample, 1 high positive sample 3 replicates within 7 days. Preferentially from extraction.
Accuracy	3 low positive samples, 3 high positive samples, 3 negative samples. If applicable, when selecting the positive samples, the genetic diversity should be taken into account. Preferentially from extraction.	10 low positive samples, 10 high positive samples, 20 negative samples. If applicable, when selecting the positive samples, the genetic diversity should be taken into account. Preferentially from extraction.
Linearity/ Measuring range/Limit of quantification	not necessary	Serial dilutions of min 5 log with 1 positive sample. 2 replicates in 2 runs. All log dilutions should be positive to be part of the measuring range.
Limit of detection/analytical sensitivity	not necessary	Can be concluded from linearity/measuring range experiment, followed by 20 measurements for lowest concentration with a confidence interval of 95 % (19/20 samples are positive)
Analytical specificity	not necessary	20 negative samples. If applicable: for microbiological tests, also analyse samples with microorganisms genetically related, unrelated but frequently detected in the same matrix, or presenting similar symptoms; for haematological tests, analyse samples from other haematological pathologies and healthy controls.

Definitions: Low positive sample= LOQ lowest concentration; High positive sample= LOQ highest concentration

It was commonly accepted that an exception to this rule can be made for some commercial CE-IVD and/or FDA certified assays. Several of these available assays are detecting a range of different amplification targets in a multiplex reaction, for instance within the GeneXpert (Cepheid) or Hemavision assays (DNA technology A/S). These assays have been extensively validated by the company during assay development and lot validation, which is guaranteed by the CE-IVD/FDA certification. Therefore, the number and nature of controls included in these certified assays can be limited. It remains however essential that these commercial assays include an internal run control to verify that the PCR-reaction is performing adequately and that instructions on transport and storage conditions are strictly followed.

The quality of the test result is also related to the amount and knowledge of the total uncertainty and trueness of the results obtained. The total uncertainty is in most cases broader than the measurement uncertainty, and needs to be estimated and documented in the validation file. In this respect we believe that determination of the inter-assay reproducibility is mandatory. Here, one could consider (if applicable) the variability due to different matrices, different reagent lots, different instruments, different environmental conditions and different operators.

A high degree of trueness for a given molecular diagnostic test can ideally be achieved when reference material is available. A laboratory may choose to relate all test results to this reference material to achieve a "true" value. It is obvious that traceability of these references is in all these cases essential. On the other hand the reference material can be used as a control material in each experiment to monitor experimental variability.

However, for most molecular diagnostic tests international standardized or reference material is lacking. In these cases, participation in inter-laboratory comparison is highly valuable to monitor trueness of molecular diagnostic tests. The choice of an External Quality Control (EQC) programme should also be well considered, since it is best to participate in an EQC programme where the pre- and post-analytical setting resembles the ones in your laboratory. Participation to the EQC programmes proposed by the WIV Institute Pasteur is mandatory for Belgian labs pursuing reimbursement by the INAMI-RIZIV.

If no EQC scheme is available, one should try to set up an informal inter-laboratory comparison between two or more laboratories, also called "ring controls".

The organisation of ring tests is one of the next objectives of the working group MolecularDiagnostics.be. Ring tests should be organised state of the art, including good data review and interpretation. It is recommended to include EQC and ring test results in the ongoing validation file of each test to monitor and improve the quality of the test on a regular basis.

7. POST-EXAMINATION PROCEDURES (§5.7)

Molecular diagnostic tests are in general technically demanding. Therefore we believe that the classical levels of reviewing and validation of examination results in the laboratory, both technical and medical, should be strictly adhered upon.

During technical validation, all data including raw and analyzed data should be available. Result entry (including results from referral labs) into the laboratory information system is most often done manually. A system to prevent transcription errors should be in place, e.g. by double-checking by a second person or by the same person at two different time points, with the original documents available. It is obvious that interpretation of test results should be performed by adequately trained personnel who apply the specific criteria for result acceptance stated in the analytical test procedure. Also, procedures should describe how to deal with non-complying run results. Finally, medical validation should correlate the obtained results with the clinical data and give authorization for release of test results. For this purpose, adequate clinical information should be made available to the laboratory.

After release of test results, medium or long term storage of the NA samples should be considered. Laboratories should reflect on this issue, debate it with the clinicians and document its policy. Storage temperatures and periods of each type of primary sample (blood, bone marrow), and other laboratory samples like DNA, cDNA, RNA and bacterial strains should be described. Most laboratories, active in haematology assays, agree that diagnostic samples are precious and availability for future development or improvement of a new test within the framework of the same diagnosis is advantageous. Samples provided for microbiological tests are in general kept for only a short or medium period. Safe disposal of samples that are no longer required for examination shall be carried out in accordance with local regulations or recommendations for waste management. Especially for molecular diagnostic assays, it is advised to take special care of waste containing PCR amplicons.

8. REPORTING OF RESULTS (§5.8)

The report should contain a clear description of the examination procedure, or refer to the (online) laboratory handbook. If the measurement procedures are important for the interpretation of the results, they may be specifically reported. This may particularly be important if different procedures are available in the laboratory with different analytical and/or diagnostic sensitivities. Also medical interpretation of the test results and factors interfering with the procedure (e.g. sample criteria) should be registered and reported. The latter should be available at the time of medical validation. If a sample is rejected for analysis, this should be mentioned in the report.

For tests which are "under development" or are in the process of validation, due to lack of positive samples (e.g. for rare translocations in haematological malignancies), results may already be reported before finalizing the validation, but this should be clearly mentioned on the report. Also, it is advised to mention either on the report or in the (online) laboratory manual which test is accredited according to an ISO standard.

For some tests in the field of molecular diagnostics, recommendations on vocabulary and syntax of reporting results (§5.8.4) have been internationally proposed and

published, either by international research groups or by official international organizations. It may be appropriate to refer to these publications and to report results according to these recommendations. Examples are the t(9;22) BCR/ABL quantitative monitoring (32-34) and the HER2 amplification analysis by FISH (35).

Finally, many procedures for reporting molecular diagnostic tests are common to those of the ('routine') clinical biology laboratory. For instance, the procedure for correction of results and for immediate or urgent notification should be described and notifications should be documented. Turn around times need to be agreed upon by laboratory management and clinicians and should be made available through the laboratory handbook. If applicable, also a procedure should exist for notification of delay.

CONCLUSION

The aim of the MolecularDiagnostics.be working group was to reach a consensus interpretation on the criteria, described in chapter 5 of the ISO15189:2007, specific for molecular diagnostic testing. For most criteria, a consensus interpretation was obtained. For pre- and post-examination storage of samples and NA, no consensus could be reached and few specific guidelines could be found in peer reviewed literature. Also the number of samples that must be used for validation was a cause for debate. Furthermore, although many EQC panels exist, the working group members expressed the value of matrix dependent testing.

The next goals of the working group will be the validation of storage conditions, organization of ring controls and standardization of specific (routine) assays.

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