DAkkS
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Guideline of the Sector Committee Pathology/Neuropathology for the validation of examination methods in Molecular Pathology

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Scope of application:

This guideline applies to all accredited pathology/neuropathology units and to those aiming for accreditation in which molecular pathological procedures are used and for which the results are used for diagnoses and decisions for appropriate therapy.

This document provides technical and scientific instructions for fulfilling the requirements for validation and verification of molecular pathological test methods. In addition, measures for validation and verification of molecular pathological test methods are elucidated which are to be newly introduced or modified.

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In this document, the male form of function designations is used as a matter of principle in the interest of legibility; it also includes the female form.

Note:

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Changes over the previous version are highlighted in yellow, or are indicated by a mark in the side margin.

Table of contents

1 Purpose / Scope of Application

This guideline elucidates the fundamentals, the performance and the documentation of the validation of test methods in the sector of molecular pathology.

This document serves as information for inspection bodies and employees of units for pathology/neuropathology.

The sections “Notes on practical implementation” merely contain explanations of a few practical examples. These sections (written in italics) do not in any way cover all the aspects to be taken into consideration for practical implementation of the methods. The specific processes to be used in actual practice remain the responsibility of the inspection agency.

2 Terms

Validation

Confirmation, through the provision of objective evidence, which the requirements for a specific intended use or application have been fulfilled (ISO 9000:2005, 3.8.5, DIN EN ISO 15189:2014).

Validation of test methods in the sector of molecular pathology

Molecular pathology is based on an algorithm-based step-wise* diagnostic mode of procedure which is an inspection activity and which contains an expert assessment (molecular pathological assessment). This is why this procedure is to be accredited according to standard DIN EN ISO/IEC 17020 in the specialist area of pathology.

Accordingly, the requirements set forth in DIN EN ISO/IEC 17025 and DIN EN ISO 15189 (ILAC-P15:06/2014) apply to the analytical steps in the diagnostic. All methods must be fully validated prior to being introduced into routine diagnostics. These methods must yield demonstrably reproducible, correct results.*

Verification

Confirmation, through provision of objective evidence, which specified requirements have been fulfilled (ISO 9000:2005, 3.8.4, DIN EN ISO 15189:2014).

* See 71 SD 4 001 Sect. 7.1

In routine diagnostics a distinction is drawn between the validation/verification of tests as described below:

“CE Tests“: “Test systems with the CE mark“ involve tests that are developed at a different agency and which are marketed as a commercial reagent kit with the CE mark. Validation of the method has been conducted by the manufacturer and the essential key performance characteristics are available.

For methods which have been validated by the manufacturer and which are applied exactly in accordance with the manufacturer’s specifications verification begins with reviewing the given key performance characteristics prior to approving the method for routine diagnostics.

“In-house Test“: In-house tests involve methods which have been developed in-house/internally by the unit for pathology/neuropathology, or which have been established on the basis of external scientific study. The unit for pathology/neuropathology is, in this case, responsible for demonstrating the suitability of the method in the corresponding application (“validation“). It may be possible to reduce the scope of validation for methods established on the basis of external scientific study.

Method validation and verification are considered to be intermeshed processes. Ongoing verification of the applied methods in routine diagnostics must be considered as being one of the most significant in-house quality assurance measures. Verification must ensure that the key performance characteristics determined over the course of method validation continue to be achieved and that the method therefore remains reproducible and stable.

Objective of Validation

The objective of method validation is to provide clear proof that the defined, specific task can be fulfilled with a given method.

Molecular pathological procedures primarily require sensitivity, specificity and correctness (recognition and avoidance of systematic errors), along with the precision of the results.

In qualitative analyses, precision applies to the reproducibility of the results (precision for repeated testing and parallel testing)).

When validating changes in the methods, statements concerning the influence of these changes (robustness of the method) are required, i.e., proof must be provided in this case that the test results do not change and are the same as for the original method.

Validation of methods consists of three vital steps:

- **Description of the molecular pathological examination procedure**

Exact description of the method in procedural instructions

- **Definition of the key performance characteristics to be determined**

Comparison of key performance characteristics with the properties required for the task at hand (quality requirements)

- **Determining of the key performance characteristics**

Documentation of proof that the quality requirements are actually fulfilled in this special case.

3 Description

3.1 Description of the molecular pathological examination procedure

The procedure represents all of the procedural steps as a whole which are required to perform the given task.

The procedure to be accredited must be fully documented so that an expert evaluator can assess the procedure without the need for any additional information. The procedure description must include at least the following information (cf. DIN EN ISO 15189; Sect. 5.5.3):

a) purpose of the examination;

b) principle and method of the procedure used for examinations;

c) performance characteristics (e.g. specification of the target region for mutation analyses);

d) Sample information (securing and shipping conditions, fresh material, formalin-fixed material, paraffin blocks, etc.);

e) Information about correct preparation of the samples (e.g. dissection);

f) required equipment and reagents;

g) environmental and safety controls (include the "Hazards and hazardous substances" instructions separately if required);

h) calibration procedure (metrological traceability);

i) procedural steps;

j) Quality assurance procedures;

k) Brief description of the process for calculating the results, or the test evaluation, including the measuring inaccuracy for the measured values (where applicable);

l) biological reference intervals or clinical decision values (where applicable);

m) instructions for determining quantitative results when a result is not within the measurement interval; (where applicable);

n) alert/critical values (where applicable);

o) Interpretation of findings;

p) potential sources of variation;

q) References (e.g., literature, software tools used, guidelines/directives).

Notes on practical implementation:

The description should always include, for example for a PCR test, an exact in-silico analysis of the target sequences in which the following questions are to be resolved:

- *Do polymorphisms exist at the primary binding sites?*
- *Do the primary sequences exhibit any further significant homologies?*
- *Do homologous target sequences exist (e.g. pseudogenes) and, if so, is their amplification discriminated?*
- *Does the amplicon include all significant areas, or which relevant mutation hotspots are detected and which, if any, are not?*

Standard databases, in addition to others, and freely accessible software tools such as BLAST, Ensembl and COSMIC can be used for this.

The record to be described is yielded from the implementation phase that precedes validation. Here, the technical parameters of the new method is tested and adapted as required so as to provide a technically stable analysis in routine operation. Validation is conducted based on this record.

3.2 Definition of the Key Performance Characteristics to be determined

The following key performance characteristics are normally applied for assessing the performance of analytical procedures: accuracy (precision, correctness) and selectivity (analytical sensitivity, specificity).

3.2.1 Accuracy

- **Precision**

Precision characterizes the extent of spread of independent analysis results on the same sample. Normally, a distinction is drawn between intra-assay precision (precision within a reaction mixture) and inter-assay precision (precision among different reaction mixtures (cf. Fig. 1).

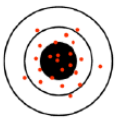
- **Correctness**

Correctness denotes the extent of concurrence of the results with the anticipated value. This can be determined in either with a previously established standard method, or using a reference population, or can be scientifically based (cf. Fig. 1).

Fig. 1: Precision and correctness: The terms "exact", "correct" or "accurate/precise" are frequently interchanged in common language. The illustrations below serve to make the distinctions between these terms clear using example of accuracy in archery.

Precise and correct
The archer shoots his arrows accurately (always on the same mark) and correctly (in the bulls eye)

Precise but incorrect
This archer also shoots his arrows accurately (always on the same mark) but not correctly (always to the right of the bulls eye)



Correct but imprecise
This archer shoots correctly (hits the target around the bulls eye) but imprecisely (clear spread or scatter of the marks hit on the target)

Incorrect and imprecise
This archer shoots incorrectly (hits the target below and to the right of the bulls eye) as well as imprecisely (clear spread or scatter of the marks hit on the target).

aus: http://www.med4you.at/laborbefunde/allgemeines/lbef_qualitaet.htm#Pr

3.2.2 Selectivity

- **Analytical sensitivity**

Analytical sensitivity is a measure of the rate of correct, positive results and describes the ratio between correct, positive results to the overall number of positive results:

Number of correct, positive / (number of correct, positive + number of incorrect, negative).

A distinction must be drawn between analytical sensitivity and the limit of detection (LOD), which is the lowest detectable amount that stands out against the background or from the negative control values (with a 95% confidence interval). Determining the limit of detection can be meaningful for individual methods.

- **Specificity**

Specificity is the measure of the rate of correct, negative results and describes the ratio between correct, negative results to the overall negative results:

Number of correct, negative/ (number of correct, negative + number of incorrect, positive).

3.3 Determining the Key Performance Characteristics

The performance characterization results, i.e., determining the individual characteristics must be documented completely and in a meaningful manner.

Characteristic key data is determined for the anticipated application area that is to be defined. Based on this characteristic key data, the following analyses are to be performed either individually or in combination with one another.

3.3.1 Determining the Accuracy Data

3.3.1.1 Determining the precision

Inter-assay precision denotes the concordance between repeated determining for different aliquots of the same sample in different reaction mixtures.

Notes on practical implementation:

It is vital that the following be clarified: Does the method generate reproducible results with the same sample with an appropriate number of different test approaches?

Example: The same sample is examined on two further days in single determination.

Intra-assay precision denotes the concordance between parallel determinations of one or more samples within a reaction mixture (an appropriate number of parallel determinations for different aliquots of the same sample).

Notes on practical implementation:

It is vital that the following be clarified: Does the method generate reproducible results with parallel determinations with an appropriate number of different aliquots of the same sample in one test approach?

Example: At least one known positive and one known negative sample is examined in a triple determination. The composition of these control items should correspond to the patient material.

With quantitative analytical methods, determination of the linear measuring range by means of spiking experiments and dilution stages is likewise recommended.

3.3.1.2 Determining the correctness

The **correctness** of a method can be established by comparing the results with those of a validated standard method, or with a validated reference population. If neither of these is available, the values which are determined must correspond to scientifically founded anticipated values.

Correctness should also be determined with an appropriate number of samples and can be combined with the process for determining the precision.

Notes on practical implementation:

As a rule, samples are used for which the test parameter has already been established through a validated method. The number of validation samples must be appropriate for availability. The composition of the validation samples should correspond to the material spectrum anticipated in the routine (e.g., DNA from FFPE tissue).

If a new method is being validated whose technical sensitivity (LOD) exceeds that of the current standard method, validation samples which were negatively tested previously can be positively tested using the more sensitive method. If all of the results of validation and the characteristics of the discrepant sample indicate the correctness of the new results, the reproducible, scientifically-founded results for the new method can be considered to be trustworthy and, consequently, the initial assessment of the sample must be corrected.

External quality assessment samples and participation in inter-laboratory tests are also suitable for checking the correctness of the method.

3.3.2 Determining the selectivity

Examination of the new method for analytical sensitivity and specificity must be conducted with an appropriate, greatest possible number of samples using known test parameters.

3.3.2.1 Determining the analytical sensitivity

Can be determined through verification testing of known positive cases

Analytical sensitivity = number of correct, positive / (number of correct, positive + number of incorrect, negative)

Notes on practical implementation:

Here, samples can be used, for example, which have been tested using a different validated standard method in a different (reference) institute.

3.3.2.2 Determining the analytical specificity

Can be determined through verification testing of known negative cases

Analytical specificity = number of correct, negative / (number of correct, negative + number of incorrect, positive)

Notes on practical implementation:

Here, samples can be used, for example, which have been tested using a different validated standard method in a different (reference) institute. Practical notes; see also 3.1

3.3.2.3 Determining the limit of detection (where required)

The LOD indicates the amount or which portion of target structures must be present in the sample to yield reliable detection (see 2.2). Both of these parameters, absolute quantity and portion, apply to the LOD. These variables are important in assessing the significance of the results of analysis of a patient sample.

Notes on practical implementation:

The LOD can be determined, for example, by means of dilution series (spiking experiments) from wild-type (wt) and mutated (mut) DNA.

The wt and the mut DNA must be set to exactly the same concentration in advance.
The concentration to be set is based on the normal values in the routine. Both samples must exhibit comparable amplification (quality) of the DNA. This can be tested with a qPCR, for example.

3.4 Approval of Validated Methods

Newly developed or adapted methods must be explicitly approved by the responsible department head (in this field a qualified scientist or specialist physician) before they can be used on the basis of the validation results. Here, a decision must be taken as to whether the quality requirements for the method have been attained and whether the method can be used for the planned tests at the inspection agency.

Proof of the qualification of the employee authorized to attest to the validation of the method and, hence, approve the method for the routine, must be provided.

3.5 Validation of Changes to the Method

For cases in which the method must be changed for analytical or organizational reasons (e.g. change of reagents), this method must be re-validated and, where required, re-approved for the routine. The scope of validation depends here on the specific change(s) made.

3.6 Verification

The approved method shall be continuously verified during ongoing operations using appropriate control mechanisms.

3.7 Documentation and Archiving

All data which has been determined for establishing the key performance characteristics of methods shall be documented and retained together with the approval note for at least five years after the last use of the method concerned.

The PCR products for the validation method and the dissected sections must be archived until the completion of validation.

3.8 External Quality Assurance Measures

External quality assurance measures include:

- Participation in suitability tests (e.g. when offered, in round-robin tests at least every 2 years)
- If no round-robin test is offered for a parameter, inter-laboratory comparisons should be performed

4 Literature

DIN ISO 5725-2:2002-12	Accuracy (trueness and precision) of measurement methods and results - Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method
DIN 32645:2008-11	Chemical analysis - Decision limit, detection limit and determination limit under repeatability conditions - Terms, methods, evaluation, 2008
DIN EN ISO 15189:2014	Medical laboratories — Requirements for quality and competence
71 SD 4 028	Guideline of the Sector Committee Pathology/Neuropathology for the validation of examination methods in immunohistochemistry
71 SD 4 019	Validation of test methods within the scope of SK – Chemistry and Environment
Process Procedure for Method Validation (Status: 02.2015)	Society for Virology