

ICS 11.100.10
C30



National Standard of the People's Republic of China

GB/T XXXX—20XX

Clinical laboratory testing and in vitro diagnostic test systems — general standard for metagenomic next-generation sequencing validation

(Draft for approval)

Issue date: 20XX-XX-XX

Implementation date: 20XX-XX-XX

Issued by State Administration for Market Regulation

Standardization Administration of the People's Republic of China

Table of contents

Foreword	2
1 Scope	3
2 Normative references	3
3 Terms and definitions	3
4 Requirements	6
4.1 Preparation of validation specimens	6
4.2 Performance validation of key testing steps	7
4.3 Performance validation of the complete process	12
4.4 Validation report	13
References	15

Foreword

SAC/TC 136 is in charge of this English translation. In case of any doubt about the contents of English translation, the Chinese original shall be considered authoritative.

This document is drafted in accordance with the rules given in GB/T 1.1 — 2020 *Directives for standardization - Part 1: Rules for the structure and drafting of standardizing documents*.

Attention is drawn to the possibility that some of the contents of this standard may be the subject of patent rights. The issuing body of this document shall not be held responsible for identifying any or all such patent rights.

This document was proposed by National Medical Products Administration.

This document was prepared by SAC/TC 136.

It is managed by the National Committee for Medical Clinical Laboratory and In Vitro Diagnostic Systems Standardization Technical Committee (SAC/TC136). The drafting units for this document include: Peking Union Medical College Hospital, Chinese Academy of Medical Sciences; National Institute for Food and Drug Control; West China Hospital, Sichuan University; The First Affiliated Hospital of Sun Yat-sen University; The First Hospital of China Medical University; National Institute of Pathogen Biology, Chinese Academy of Medical Sciences & Peking Union Medical College; Institute of Microbiology, Chinese Academy of Sciences; Zhujiang Hospital, Southern Medical University; Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China; The First Affiliated Hospital of Guangzhou Medical University; The First Affiliated Hospital, Zhejiang University School of Medicine; National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention; Huashan Hospital, Fudan University; Beijing Institute of Medical Device Testing (Beijing Medical Biological Protection Equipment Testing Research Center)

The primary drafters of this document are: Xu Yingchun, Huang Jie, Yang Qiwen, Chen Bojiang, Chen Peisong, Zhang Dong, Liu Donglai, Wang Jing, Wang Ruizhi, Ren Shanshan, Han Xiaoxu, Ren Lili, Wu Linhuan, Guan Wenda, Jiang Li, Zhou Haijian, Zhang Wenhong, Zhou Hongwei, Chen Dingqiang, Zhang Jie, Xiao Yan, Yang Zifeng, Jiang Juan, Ai Lu, Dong Xiaolong, Han Dongsheng, Zhao Qianwen, Zuo Liyuan, Lu Xin, Hu Jinrui, Wang Huiru, Zou Yingshu.

Clinical laboratory testing and in vitro diagnostic test systems — general standard for metagenomic next-generation sequencing validation

1 Scope

This document specifies the requirements for performance validation that clinical laboratories must conduct prior to implementing metagenomic next-generation sequencing (mNGS).

This document is applicable to clinical laboratories utilizing high-throughput sequencing technologies for mNGS performance validation.

This document does not apply to the performance validation of single-molecule pathogen sequencing technologies primarily based on biological nanopores, solid-state nanopores, etc., nor to pathogen sequencing performance validation established through targeted probe hybridization capture or multiplex primer amplification technologies.

2 Normative references

This document contains no normative references.

3 Terms and definitions

The following terms and definitions are applicable to this document.

3.1

next-generation sequencing; NGS

A DNA sequencing technology characterized by the parallel determination of hundreds of thousands to millions of nucleic acid sequences in a single run, typically with shorter read lengths.

[Source: GB/T 30989-2014, 3.19, with modifications]

3.2

metagenomic next-generation sequencing mNGS

A detection technology that performs unbiased sequencing of all microbial DNA and/or RNA present in a specimen.

[Source: ISO/TS 24420:2023, 3.23, with modifications]

3.3

validation

The process of providing objective evidence that specific intended uses or application requirements have been met.

[Source: GB/T 19000-2016/ISO 9000:2015, 3.8.13, with modifications]

3.4

read length of gene sequencing

The length of quality-assured sequence fragments that can be read in a single run, typically expressed in base pairs.

[Source: GB/T 30989-2014, 3.24, with modifications.]

3.5

base calling

The process during sequencing where fluorescent signals or other signals generated by the sequencing reaction are converted into sequence information.

[Source: GB/T 30989-2014, 3.26]

3.6

quality of base calling

A measure of the probability that a base has been correctly identified, typically represented directly by ASCII codes.

[Source: GB/T 30989-2014, 3.29]

3.7 library

A reconstructed collection of molecules obtained through biological sources, artificial synthesis, cloning techniques, etc., such as genomic libraries or complementary DNA libraries.

[Source: GB/T 30989-2014, 3.5, with modifications]

3.8 bioinformatics

An interdisciplinary field that applies methods and technologies from information science and related disciplines to study and analyze the storage, processing, and transmission of information in biological systems and biological processes.

[Source: GB/T 29859-2013, 2.1.1]

3.9 reads per million

The number of sequences aligned to the target species' genome per one million sequencing reads.

3.10 coverage rate

The proportion of sequences aligned to the target species' genome relative to the reference genome of that species. Coverage rate (%) = (Length of Covered Regions / Total Length of Reference Sequence) × 100%.

[Source: GB/T 30989-2014, 3.30, with modifications]

3.11 relative abundance

The proportion of a particular microbial species within its corresponding classification (typically divided into four major categories: bacteria, fungi, viruses, and parasites).

[Source: ISO/TS 24420:2023, 3.19, with modifications]

3.12 closely related species

Species that are closely related on the evolutionary tree.

3.13 cut-off value

In qualitative assays, the critical point refers to a specific point in the detection reaction below which the qualitative detection result is judged as negative, and above which it is judged as positive.

[Source: WS/T 505-2017, 3.13, with modifications]

3.14 background laboratory contamination

Microbial nucleic acids, other than those of the target microorganism, may originate from reagents, consumables, personnel, laboratory space, and other sources.

3.15 precision

The degree of agreement between measurement readings or measured values obtained by repeated measurements on the same or similar test objects under specified conditions.

[Source: GB/T 29791.1-2013, A.3.29]

3.16 repeatability

The precision under repeatability conditions, including the same measurement procedure, same operator, same measurement system, same operating conditions, and same location, with repeated measurements of the same or similar specimens in a short time frame.

[Source: GB/T 29791.1-2013, A.3.30]

3.17 intermediate precision

Measurement precision under a set of measurement conditions, which include the same measurement procedures, the same location, and repeated measurements of the same or

similar test objects over an extended period of time, but may include changes in other related conditions.

[Source: GB/T 29791.1-2013, A.3.20]

3.18

detection limit

limit of detection

LoD

A testing procedure's lowest measurable value that can be reported as present at a specific confidence level, also referred to as the minimum detectable concentration. LoD is typically expressed as LoD_{95%}, indicating a 95% probability of correctly detecting the pathogen concentration.

Note: Analytical sensitivity is sometimes used to denote the limit of detection.

[Source: GB/T 29791.1-2013, A.2.7 and A.2.8, with modifications]

3.19

stability

The ability of reagent components to maintain their performance characteristics under specified storage conditions, freeze-thaw cycles, and time limits.

[Source: GB/T 29791.1-2013, 3.68, with modifications]

3.20

analytical specificity

The capability of the measurement system, using a specified measurement procedure, to provide measurement results for one or more measurands that are mutually independent and independent of any other quantities in the system under measurement.

Example: The ability of a measurement system to determine plasma creatinine concentration using an alkaline picrate method without interference from glucose, uric acid, ketones, or proteins.

Note: The specificity of the measurement procedure should not be confused with diagnostic specificity.

[Source: GB/T 29791.1-2013, A.3.4]

3.21

interfering

Changes in test results caused by other components or characteristics of the sample.

[Source: WS/T 408-2024, 3.6]

3.22

cross-reactivity

Using a paired comparison method to determine the impact on detection results when nucleic acid fragments of homologous or closely related pathogens are present simultaneously or individually.

3.23

accuracy

The degree of closeness between the measurement result and the true value of the analyte.

[Source: WS/T 505-2017, 3.4]

3.24

sensitivity

true positive rate

The proportion of positive diagnostic test results or counts exceeding the decision threshold among patients with a clearly defined clinical disease, reflecting the ability of the new test to correctly determine whether a patient has a certain disease.

[Source: WS/T 505-2017, 3.9]

3.25

specificity

true negative rate

The proportion of negative diagnostic test results or counts within the decision threshold among patients without a specific clinical disease, reflecting the ability of the new test to correctly exclude a certain disease.

[Source: WS/T 505-2017, 3.10]

4 Requirements

4.1 Preparation of validation specimens

Laboratories shall strictly adhere to the specimen types and pathogen spectra involved in the intended clinical applications, separately prepare validation specimens, and immediately store them at a stable temperature until the day of performance validation testing. Based on different preparation processes, validation specimens may be categorized into three forms: clinical specimens, simulated positive specimens, and computer-simulated sequencing data.

a) Clinical specimens: the ideal validation specimens are the clinical specimens intended for the expected clinical applications. When laboratories are unable to obtain sufficient residual positive clinical specimens representing various types of pathogens at appropriate concentrations, simulated positive specimens may be used to complete performance validation. For clinical intended uses aiming to detect pathogen resistance genes, validation specimens shall be prepared according to the range of resistance genes being tested, using clinical resistance phenotypes or other resistance gene detection methods as comparative methods to confirm relevant analytical performances.

b) Simulated positive specimens: these cannot be used to establish cut-off values or confirm accuracy performance. To avoid matrix effects, simulated positive specimens shall use residual negative clinical specimens with clearly defined negative test results as the dilution matrix. Additionally, changes in host cell concentration can affect the LoD of mNGS. During initial performance validation, laboratories should use specimens with common host cell concentration levels to preliminarily establish LoD and other performance indicators. According to the specimen volume required by the performance validation plan, traceable or quantified residual positive clinical specimens, standard microbial strains, etc., should be mixed into residual negative clinical specimens to simulate positive specimens. If laboratories intend to perform performance validation on free DNA detection processes, pathogen nucleic acids with fragment length distribution and concentration consistent with positive clinical specimens shall be added to residual negative clinical specimens.

c) Computer- simulated sequencing data: used solely for the performance validation of bioinformatics analysis processes. For species within the pathogen spectrum that are difficult to obtain true clinical positive specimens or standard microbial strains/toxins, simulated sequencing data can be generated by adding simulated pathogen sequencing data to previously negative sequencing data. The simulation of pathogen sequencing data involves two main steps:

- 1) Download high-quality reference genomes from databases and perform data cleaning and fragmentation.
- 2) Use software to simulate sequencing data, setting relevant parameters based on actual sequencing data conditions, such as sequence length of 75 bp and sequencing error rate of 1/1000.
- 3) Finally, the sequencing data from clinical specimens and computer-simulated sequencing data are mixed to form bioinformatics performance validation specimens. These should cover all pathogens within the pathogen spectrum and include different sequence gradients (e.g., 10,000; 1,000; 100; 10; 1 sequences) and proportions (e.g., 1:0, 1:1, 1:3) of closely related pathogen sequences.

4.2 Performance validation of key testing steps

4.2.1 Performance validation of specimen collection, transportation, and storage

Standardizing specimen collection and transportation to ensure that testing specimens are qualified is a prerequisite for accurate test results. It shall be ensured that personnel collecting specimens have easy access to specimen collection manuals and can correctly implement the manual's requirements. Specimen collection containers, transportation

methods, and time limits shall be clearly specified to ensure that medical staff and specimen transport personnel receive accurate information regarding specimen collection and transportation before specimen collection. If laboratories or clinical departments intend to use different collection containers, performance validation of specimen collection, transportation, and storage for each type of container shall be completed separately.

Should adopt specialized transport containers, selecting appropriate specimen transport containers based on different transportation distances and environmental conditions, and monitor and record the specimen location and storage temperature during the transportation process. When transporting specimens within the institution, specimens shall be packaged in specimen bags with biohazard labels to prevent contamination of other specimens and transport containers in the event of specimen leakage. When transporting specimens outside the institution, if the specimen is confirmed or suspected to be a high-pathogenicity microorganism (toxin) species or specimen, relevant transport permits shall be obtained. Upon receiving specimens, the laboratory shall immediately evaluate the quality of the specimens, which should include unique specimen identification, specimen quantity, and transport conditions.

Fresh specimens should be selected for nucleic acid extraction; if immediate testing cannot be guaranteed, specimens shall be promptly aliquoted and properly stored according to their characteristics. Different specimen types and pathogens have varying pre-treatment methods and stability times. Laboratories should consider the storage conditions and testing frequency of specimens in actual clinical applications to perform performance validation of specimen stability.

- a).. Performance validation method: use prepared weak positive specimens and negative specimens, storing them under different temperatures (e.g., -80°C, -20°C, 4°C, room temperature), different storage durations (e.g., 1 day, 3 days, 7 days), and different freeze-thaw cycles (e.g., 1, 2, 3 times). Utilize laboratory-validated quantitative fluorescent PCR techniques (qPCR, ddPCR, etc.) or the intended in vitro diagnostic systems to perform three repeated tests for each specimen under each storage condition.
- b).. Analysis: Determine storage conditions with compliance rates of $\geq 95\%$, establishing the performance of specimen stable transportation and storage temperatures, durations, and freeze-thaw cycles.

Note 1: For more information on clinical microbiology specimen collection, refer to WS/T 640-2018.

Note 2: For more information on specimen cold chain logistics transportation, refer to GB/T 42186-2022.

Note 3: For more information on laboratory specimen reception and handling, refer to GB/T 42060-2022.

4.2.2 Performance validation of specimen pre-treatment and nucleic acid extraction reagents

Select pre-treatment and nucleic acid extraction processes based on the types of specimens to be tested, primarily including grinding, liquefaction, host depletion, lysis, and purification steps. Before clinical application, performance validation shall be conducted on the yield, purity, integrity of the pre-treatment and nucleic acid extraction reagents intended for use, as well as their repeatability and inter-batch variability.

- a) Performance validation method: preferably use standard substances consistent or similar to the specimen types. When suitable standard materials are not available, clinical specimens prepared according to 4.1 or simulated clinical positive and weakly positive specimens may be used.
 - 1) Using the established process, within the same laboratory, by the same operator, using the same equipment and batch of nucleic acid extraction reagents, repeatedly extract no fewer than six parallel specimens within one day.
 - 2) Using the established process, within the same laboratory, by different operators, using the same equipment and different batches of nucleic acid extraction reagents, repeatedly extract no fewer than three parallel specimens on different days.

- b) Analysis: determine the yield (given the significant variation in pathogen content in mNGS-tested specimens and the differing extraction efficiencies of reagents for various pathogen types, quantitative fluorescent PCR techniques should be used), purity, integrity, as well as repeatability and inter-batch variability.

Note: For more information on performance validation of nucleic acid extraction and purification reagents used in laboratories, refer to GB/T 37875-2019.

4.2.3 Performance validation of library preparation reagents

The quality of the library is crucial for the quality of sequencing data. The fragment length distribution and concentration of the library shall meet the sequencing requirements of high-throughput sequencers. Different laboratories may use varying library preparation processes and reagents [reverse transcription (RNA processes), purification, nucleic acid fragmentation (including but not limited to enzymatic digestion, sonication, etc.), end repair, adapter ligation, PCR amplification, library fragment selection and purification, etc.], which shall be clarified before performance validation within the same laboratory. Confirm the fragment length distribution, purity, concentration, and total quantity of the prepared library, and perform performance validation on the repeatability and inter-batch variability of the library preparation reagents.

- a) Performance validation method: utilize specimens that have completed performance validation for pre-treatment and nucleic acid extraction processes, extract nucleic acids from validation specimens prepared in 4.1, and proceed with library preparation.
 - 1) Using the established process, within the same laboratory, by the same operator, using the same equipment and batch of reagents, repeatedly prepare libraries for no fewer than six parallel specimens within one day.
 - 2) Using the established process, within the same laboratory, by different operators, using the same equipment and different batches of reagents, repeatedly prepare libraries for no fewer than three parallel specimens on different days.
- b) Analysis: use available methods to confirm the fragment length distribution, purity, concentration, and total quantity of the library, and refer to the calculation methods in 4.2.2 to determine repeatability and inter-batch variability.
 - 1) Library length distribution: use fragment length analyzers to assess library length. Repeated library preparations should exhibit a single, smooth peak close to a normal distribution. Determine the length distribution range and average library length based on the results.
 - 2) Library purity: ultraviolet spectrophotometry may be used to determine the optical density (OD_{260} , OD_{280} , OD_{230}) at 260 nm, 280 nm, and 230 nm, calculate the OD_{260}/OD_{280} and OD_{260}/OD_{230} ratios, and confirm the purity of the library.
 - 3) Library concentration and total quantity: ultraviolet spectrophotometry, fluorescent quantification, or other methods may be employed to determine the library concentration and calculate the total quantity of the library.

4.2.4 Performance validation of sequencing instruments and reagents

Different sequencing platforms have unique parameters, including instrument size, throughput, read length, run time, and sequencing depth. Laboratories shall select appropriate sequencing instruments based on clinical application needs. Performance validation shall be conducted for sequencing coverage rate, average sequencing depth, sequencing accuracy, and repeatability of sequencing instruments and reagents.

- a) Performance validation method: use specimens, standard strains, or reference materials with stable sources, complete traceability information, and known reference sequences. Follow the sequencing sample preparation processes and sequencing operations provided by the sequencing instrument manufacturer to process and quality control sequencing specimens, and perform sequencing three times.
- b) Analysis: determine sequencing coverage rate, average sequencing depth, sequencing accuracy, and repeatability.

Note: For more information on performance validation of sequencing instruments and reagents, refer to YY/T 1723-2020.

4.2.5 Performance validation of bioinformatics analysis processes

Bioinformatics analysis processes are a critical component of mNGS. Laboratories shall ensure that bioinformatics analysis software meets clinical intended uses and identify any defects and potential risks associated with the software. Performance validation shall at least include the following three aspects:

- Based on the pathogen spectrum range, simulate single-species positive sequencing data with different sequence gradients to confirm the species identification capability of the bioinformatics analysis process within the intended reporting range.
 - Simulate positive sequencing data for evolutionarily closely related species and analyze them after mixing at different proportions to confirm the cross-interference rate of closely related species within the bioinformatics analysis process.
 - Laboratories shall use a statistically significant number of subject specimens, which can be based on sequence counts, relative abundance values, or ratios with blank controls and/or negative quality controls. By establishing Receiver Operating Characteristic (ROC) curves, determine the cut-off points for each species. Cut-off points should consider laboratory background contamination, including intra-batch and inter-batch laboratory background contamination. Intra-batch laboratory background contamination may originate from specimen collection processes, aerosol contamination between specimens during testing, laboratory environments, or homologous sequences of closely related species. Monitor background contamination for each batch using blank controls and/or negative quality controls, and record and analyze these during report interpretation.
 - If contamination caused by the specimen collection process is considered, resampling or the collection of corresponding control specimens shall be performed for re-testing.
 - If aerosol contamination between specimens is considered, it shall be regarded as a failure of specimen quality control, and re-testing or re-sampling followed by testing shall be performed.
 - If laboratory environmental contamination is considered, other methodologies, such as qPCR, should first be used to verify the species, and recent detection data of related species should be referred to. If necessary, promptly update the critical thresholds for the relevant species.
 - If false positives are considered to be caused by homologous sequences of closely related species, it should be determined that the detected sequences can be accurately matched to the species. Refer to cross-interference rate data of closely related species during performance validation to ascertain whether it is a confirmed detection. If necessary, use other methodologies, such as qPCR, to verify the species.
- Monitoring of inter-batch laboratory background contamination can primarily be conducted in the

following two aspects

— Before changing reagent batches, batch comparisons should be performed on the new reagent

batches to identify changes in laboratory background contamination caused by batch changes.

— Continuously monitor changes in laboratory background contamination, and regularly

communicate with clinical teams and analyze data to clarify the detection performance of

different species. If a decline in the detection performance of specific species is observed, analyze

the reasons and determine appropriate solutions.

a) Performance validation method: Fixed versions of bioinformatics analysis software and databases shall be used to perform bioinformatics analysis on computer-simulated sequencing data prepared in section 4.1 and sequencing data from real clinical specimens. Specimens or data included in the study shall originate from representative populations relevant to the application of the detection project results, and detection results shall be determined by laboratory and/or clinical standards. If defects in the analysis software are identified during the performance validation process, they shall be promptly patched. Major updates to the analysis software shall follow the principle of high risk. It should use performance validation data and clinical specimen sequencing data to reconfirm the laboratory background contamination, critical thresholds, and other performance aspects of the detection system before updating.

b) Analysis:

1) Species identification capability: the laboratory shall calculate and confirm accuracy, recall,

precision, and F1 score (the harmonic mean of precision and recall) in accordance with Formulas

(1) to (4).

$$A = (T_P + T_N) / (T_P + F_N + F_P + T_N) \dots\dots\dots(1)$$

$$R = T_P / (T_P + F_N) \dots\dots\dots(2)$$

$$P = T_P / (T_P + F_P) \dots\dots\dots(3)$$

$$F_1 = 2T_P / (2T_P + F_P + F_N) \dots\dots\dots(4)$$

Where:

A — Accuracy

T_P — True Positives

T_N — True Negatives

F_N — False Negatives

F_P — False Positives

R — Recall

P — Precision

F_1 — F1 Score

2) Cross-reactivity of closely related pathogens: analyze and calculate the cross-reactivity rates

among different closely related species based on statistical analysis results. Use these cross-

reactivity rates to define the range of proportions for determining definite detection of a species

when two closely related species are present in the analysis results.

3) Method for establishing critical points: statistically analyze the results to calculate the sensitivity

[Formula (5)] and specificity [Formula (6)] of species at different critical points. Plot the variables

of 1-specificity and sensitivity on the x-axis and y-axis respectively, mark each point on the Cartesian coordinate system, and connect them to form a curve (i.e., ROC). Select the critical point corresponding to the maximum value of the correct index (also known as Youden's index) [Formula (7)] as the optimal critical point.

$$S_E = T_P / (T_P + F_N) \times 100\% \dots \dots \dots (5)$$

$$S_P = T_N / (T_N + F_P) \times 100\% \dots \dots \dots (6)$$

$$Y_I = (S_E + S_P) - 1 \dots \dots \dots (7)$$

Where:

S_E — Sensitivity

S_P — Specificity

Y_I — Youden's Index

Note 1: For more information on performance validation of bioinformatics analysis processes, refer to ISO/IEC TS 4213-2022.

Note 2: For more information on establishing cut-off points using ROC curves, refer to CLSI GP10-A-1995.

4.3 Performance validation of the complete process

4.3.1 Precision

Precision metrics, including repeatability (intra-batch) and intermediate precision (intra-laboratory), shall be validated for the testing system using standard deviation or coefficient of variation. If laboratory test results only indicate whether the pathogen is negative or positive and do not include quantitative values such as the number of species sequences, relative abundance, and coverage, reproducibility can be assessed only.

- a) Performance validation method: using reagents from the same batch, test positive specimens, weak positive specimens, and negative specimens in at least five batches, with each batch repeating complete testing of each type of specimen at least three times.
- b) Analysis: for repeatability performance validation, calculate whether the conformity rate for positive and negative results exceeds 95%. For intermediate precision performance validation, use general office software to calculate the mean and standard deviation of each specimen's test results (parameters corresponding to cut-off points) across batches, calculate the overall mean of batch means (total mean), average intra-batch standard deviation, inter-batch standard deviation, and intra-laboratory standard deviation.

Note: For more information on precision, refer to YY/T 1789.1-2021.

4.3.2 LoD

Performance validation should be conducted for the LoD of different species within the testing system. Given the high cost of mNGS testing, laboratories may preliminarily determine the LoD concentration range by testing a small number of gradient-diluted specimens at different concentrations and then perform sufficient repeated tests on specimens at corresponding concentrations to confirm the LoD.

- a) Use traceable standard substances, cultures, or positive specimens mixed into negative specimens to prepare gradient-diluted positive specimens.
- b) Each gradient-diluted specimen shall be tested no fewer than three times, recording the detection (or positive) results at different dilution/concentration levels.
- c) Based on the data characteristics of the test results, apply appropriate statistical models and analysis methods (e.g., Probit analysis) to calculate the pathogen concentration at a 95% positive detection rate level, determining the preliminary LoD concentration range.
- d) Prepare positive specimens near the LoD concentration level and perform repeated testing on no fewer than 20 specimens at the LoD concentration level to determine the LoD for different species.

Note: For more information on LoD, refer to YY/T 1789.3-2022.

4.3.3 Stability

In the mNGS testing process, different reagent components shall be utilized. The storage instructions for the reagent components used shall be referenced, and based on actual conditions, the stable storage conditions for the reagents used shall be defined through a stability performance validation plan.

- a) Performance validation method: refer to the reagent manufacturer's instructions, repeatedly freeze-thaw and store the reagent components up to the number of times or duration specified in the reagent instructions. Subsequently, test positive specimens, weak positive specimens, and negative specimens.
- b) Analysis: determine storage conditions with conformity rates of $\geq 95\%$ to establish the stable storage conditions for reagent stability.

Note: For more information on stability, refer to YY/T 1579/ISO 23640.

4.3.4 Analytical specificity

4.3.4.1 Interference testing

Interfering substances generally include endogenous substances (such as hemoglobin, triglycerides, bilirubin, etc.) and exogenous substances (such as drugs, anticoagulants, etc.). Studies should utilize interfering substances and concentrations that may realistically be present.

- a).. Performance validation method: repeatedly test positive specimens and weak positive specimens with added interfering substances at least three times.
- b).. Analysis: determine conformity rates of $\geq 95\%$ to clarify the extent to which different interfering substances affect test results.

4.3.4.2 Cross-reactivity

Based on the clinically intended pathogen spectrum coverage, select representative positive specimens of closely related species, such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Candida albicans* and *Candida tropicalis*, to determine the cross-reactivity rates of closely related species.

- a) Performance validation method: closely related species shall be mixed separately according to similar concentrations and a high-low concentration ratio, and then detected.
- b) Analysis: the laboratory shall statistically analyze the number of sequences detected or indicators such as relative abundance to determine the cross-interference rate of closely related species.

Note: For more information on analytical specificity, refer to YY/T 1789.5-2023.

4.3.5 Accuracy

Accuracy performance validation should preferably use standard methods as reference methods, including but not limited to culture, PCR, first-generation sequencing, etc. When no standard reference methods are available, clinical pathogen diagnosis can be used as the reference method. Each category of pathogens should have a certain number of positive cases, and healthy human specimens should not be used. Considering the complexity of the methodology and the randomness of clinical positive specimens, performance validation shall be conducted on species within the reporting range during the initial performance confirmation; during clinical application, the reporting range may be continuously monitored and expanded.

- a) Accuracy performance validation method: clinical specimens should be continuously detected in parallel using mNGS and comparative methods, with the comparative methods obtaining at least 50 positive specimens and 50 negative specimens. Specimens with discrepant results between mNGS and comparative methods may be clarified using the "gold standard" or "reference method" .
- b) Analysis: calculate the sensitivity/true positive rate [Formula (5)] and specificity/true negative rate [Formula (6)] of the detection reagents according to the formulas.

Note: For more information on accuracy, refer to WS/T 505-2017.

4.4 Validation report

4.4.1 Intended use

The validation report shall clarify whether the detection system employed meets the intended purposes set prior to validation based on the validation results. It shall reflect the types of specimens and pathogen detection performances that have undergone validation, serving as the reporting range with confirmed detection performances. If the detection performance for certain pathogens does not meet the expected performance, the laboratory should continue to modify and optimize the detection system to complete validation or remove them from the reporting range.

During clinical application, the laboratory shall regularly review clinical case data, calculate the concordance rate between test results and clinical diagnoses, analyze potential issues with detection results, identify previously unanticipated risks, and optimize the detection process to improve accuracy and reliability. Additionally, laboratories are encouraged to gradually expand the applicable range based on actual clinical needs after completing validation for more pathogens and specimen types.

4.4.2 Testing system

The validation report shall include detailed information about the detection system used, including the sequencer, nucleic acid extraction reagents, library preparation reagents, sequencing reagents, version numbers of bioinformatics analysis software and databases, specimen types, pathogen spectrum, standard operating procedures, etc.

4.4.3 Raw data

In principle, the validation report shall include all original experimental records, calculation processes, and experimental results, objectively displaying the performance parameters of the detection system used. Sequencing data, original data from analysis processes, and record texts shall be saved in read-only file formats on designated computers or servers, and the storage locations shall be indicated in the validation report for easy retrieval.

4.4.4 Limitations

The limitations of the detection system in the validation report shall include at least two aspects: pathogens and specimen types.

- For certain pathogens within the pathogen spectrum, if complete validation cannot be achieved due to insufficient positive clinical residual specimens, they shall be regarded as pathogens that cannot be accurately detected. For suspicious positive specimens, other detection methods (such as PCR, Sanger sequencing, etc.) should be used for verification before reporting.
- If the laboratory intends to conduct clinical testing for multiple specimen types, validation should be carried out separately for each specimen type, and specimen types for which validation has not been completed shall be regarded as specimen types that cannot be accurately detected.

Bibliography

- [1] GB/T 19000-2016/ISO 9000:2015 Quality management systems — Fundamentals and vocabulary.
- [2] GB/T 29791.1-2013 In vitro diagnostic medical devices — Information supplied by the manufacturer (labelling) — Part 1: Terms, definitions and general requirements.
- [3] GB/T 29859-2013 Bioinformatics terms.
- [4] GB/T 30989-2014 Technical regulation of high-throughput gene sequencing.
- [5] GB/T 37875-2019 General rules for evaluation of nucleic acid extraction and purification methods.
- [6] GB/T 42060-2022 Medical laboratories — Requirements for collection, transport, receipt, and handling of samples.
- [7] GB/T 42186-2022 Operation specification for drug cold chain logistics.
- [8] WS/T 505-2017 Guideline for evaluation of qualitative test performance.
- [9] WS/T 640-2018 Specimen collection and transport in clinical microbiology.
- [10] WS/T 408-2024 Guidelines for Performance Verification of Quantitative Testing Procedures.
- [11] YY/T 1579/ISO 23640 In vitro diagnostic medical devices — Evaluation of stability of in vitro diagnostic reagents.
- [12] YY/T 1723-2020 High-throughput gene sequencer.
- [13] YY/T 1789.1-2021 In vitro diagnostic test systems — Performance evaluation method — Part 1: Precision.
- [14] YY/T 1789.3-2022 In vitro diagnostic test systems — Performance evaluation method — Part 3: Limit of detection and limit of quantitation
- [15] YY/T 1789.5-2023 In vitro diagnostic test systems — Performance evaluation method — Part 5: Analytical specificity
- [16] ISO/TS 24420: 2023 Biotechnology — Massively parallel DNA sequencing — General requirements for data processing of shotgun metagenomic sequences
- [17] ISO/IEC TS 4213-2022 Information technology — Artificial intelligence — Assessment of machine learning classification performance
- [18] CLSI GP10-A-1995 Assessment of the Clinical Accuracy of Laboratory Tests Using Receiver Operating Characteristics (ROC) Plots; Approved Guideline
- [19] National medical products administration. Guidelines for technical review of chlamydia trachomatis and/or neisseria gonorrhoeae nucleic acid detection kits [R] 2021
- [20] National medical products administration. Guidelines for technical review of aspergillus nucleic acid detection kits [R] 2022
- [21] National medical products administration. Guidelines for technical review of mycobacterium tuberculosis complex nucleic acid detection kits [R] 2023
- [22] National medical products administration. Guidelines for technical review of toxoplasma gondii, rubella virus, cytomegalovirus, and herpes simplex virus antibody and immunoglobulin G antibody avidity test kits [R] 2024
- [23] National medical products administration. Guidelines for technical review of monkeypox virus nucleic acid detection kits [R] 2024
- [24] Bharucha T, Oeser C, Balloux F, et al. STROBE-metagenomics: a STROBE extension statement to guide the reporting of metagenomics studies[J] Lancet Infect Dis 2020, 20(10): e251-e260 DOI: 10.1016/S1473-3099(20)30199-7
- [25] Department of Health and Human Services Food and Drug Administration. Infectious Disease Next Generation Sequencing Based Diagnostic Devices: Microbial Identification and Detection of Antimicrobial Resistance and Virulence Markers; Draft Guidance for Industry and Food and Drug Administration Staff; Availability[R] FDA-2016-D-0971 2016
- [26] Zhang, D., Zhang, J., DU, J., et al. (2022). The scheme for validation of clinical metagenomics sequencing assay. Chinese Journal of Laboratory Medicine, 899-905. DOI: 10.3760/cma.j.cn114452-20220721-00426