ACCREDITATION SCHEME FOR LABORATORIES

Technical Note MED 002

Specific Criteria for Molecular Pathology Section

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1. Introduction & Scope

1.1 This document describes the specific requirements for Molecular Pathology.

1.2 The document shall be read in conjunction with ISO 15189 Medical laboratories – Requirements for quality and competence', SAC-SINGLAS documents, Proficiency Testing Technical Note 001, and other MEDICAL Series Technical Notes published by SAC-SINGLAS.

1.3 In this document, molecular pathology testing refers to the detection and analysis of targeted nucleic acids (DNA or RNA) from patient samples. The scope of testing includes infectious disease, oncology (diagnosis, prognosis, therapeutic guidance, residual disease detection), heritable diseases, genetic identity testing (forensic, paternity) and pharmacogenomics

1.4 Infectious diseases

1.4.1 Molecular diagnostic techniques are used to detect the presence of infectious organisms, quantify pathogens in blood or other specimens, study the relationship among organisms from an infectious outbreak, and identify clinically relevant characteristics of pathogens. These techniques include the detection of nucleic acid through amplification, hybridization and nucleic acid sequencing.

1.5 Genetic diseases / disorders

1.5.1 In the post-genomic era (after completion of the Human Genome Project in early 2000s), molecular diagnostic tests will be used not only for classic single-gene genetic diseases, but also for complex disease or disorder or traits such as asthma, diabetes, atherosclerotic heart disease, and most other medical conditions predicted by family history.

1.6 Cancers and oncological risk assessment

1.6.1 Understanding the underlying genomic changes that cause cancer has led to the development and use of molecular diagnostic tests for the diagnosis, prognosis and monitoring disease following treatment of cancers. Molecular diagnostic techniques are used for leukaemias, lymphomas, sarcomas and carcinomas.

1.7 Genetic identity testing

1.7.1 Methods for DNA fingerprinting were first developed for forensic investigations but are now widely used in medical testing related to bone marrow transplantation, genetic assessment of hydropic and molar pregnancies, and maternal contamination analysis for prenatal genetic testing, as well as paternity testing.

2. Facilities and Environmental Conditions

The design and use of facilities in a molecular laboratory will vary depending on methods, objectives and risks.

3. Pre-examination Procedures

3.1 Sample Processing

3.1.1 Refer to Pre-Examination Procedures in **General Technical Note: Medical – 001**. In addition to that the following is applicable to Molecular Pathology laboratory.

3.1.2 Amplification procedures should be designed to minimize carryover using appropriate physical containment and procedural controls. Reagents must be prepared and aliquoted in an area (Pre-PCR or clean area) separate from the specimen preparation and post-amplification areas (dirty areas). There should be a unidirectional workflow from “clean” to “dirty” areas, with change of laboratory coats and gloves where appropriate.

If aliquoting of samples is performed, there is a written procedure that prevents cross-contamination of the aliquot containers (e.g. that no aliquot is ever returned to the original container).

3.1.3 Sample identification should be traceable through all phases of analysis, such as specimen receipt, nucleic acid extraction and quantification, endonuclease digestion, amplification, PCR product purification and dilution, electrophoresis, photography, sequence file/folder destination and naming, sequence analysis and storage of sample, result, sequences and other data.

If residual samples are used for amplification-based testing, policies and procedures ensure absence of cross-contamination of samples. This applies, for example, when a liquid based cytology sample is tested (post-cytologic processing) for *C. trachomatis* or *N. gonorrhoea* by nucleic acid amplification methods. Samples obtained from tubes on automated tracks for multiple analytes may also have risk of cross-contamination.

3.1.4 For paraffin-embedded tumour specimens from which DNA is extracted for analysis (e.g. microsatellite instability, KRAS or KIT analysis), there is a record of histological assessment of neoplastic cell content.

When performing molecular testing for the detection of *M. tuberculosis (MTB)* directly from clinical specimens, culture should be performed on all samples regardless of the molecular test result. The MTB culture may be performed in the same or different laboratory, and period correlation between molecular tests and culture should be done.

3.1.5 Nucleic acids are obtained by sample concentration, extraction and purification by methods reported in the literature, by an established commercially available kit or instrument, or by a validated method developed by the laboratory. The nucleic acid extraction method used should be compatible with specimen type and the amplification method, and any change of extraction method should be assessed for impact on overall test performance.

3.1.6 The quantity, integrity and purity of nucleic acid is measured, when appropriate. This is especially important in RNA assays due to the lability of RNA.

3.1.7 Ribonuclease-free conditions are maintained for all assays that detect RNA or use an RNA probe.

3.1.8 Linearity data for quantitative tests are verified at specified periodic intervals, not to exceed one year or manufacturer’s recommendations.

3.1.9 The whole process should be assessed for possibility of contamination either during sample processing, PCR reaction and carry-over. There should be a system to detect potential false-positives and to investigate suspect cases in a systematic manner.

4. Examination Procedures

4.1 Validation of Test Performance

4.1.1 Test validations should be performed before new tests are introduced for clinical use, including nucleic acid extraction if performed on an open platform. The procedure for such validation should be documented. The performance of new tests may be validated by comparison with reference methodologies (e.g. culture or western blot assays). If this is not possible, NAT results may be confirmed by hybridization, sequencing of amplified products, or by using alternative NATs. Participation in external proficiency testing or split samples for inter-laboratory comparison may also be used. In situations where all these are not possible, using specimens from patients with clinically documented infections may be permitted for validation of NAT results. For uncommon diseases, ongoing and progressive evaluation may need to be done after implementation of test.

For infectious disease, laboratory-developed tests should be validated with both negative and positive materials, and for cross-reactivity. Material to be used may include: synthetic DNA or RNA, culture extract, clinical specimens, product sequencing, use of EQA material.

4.1.2 Commercial test kits, including nucleic acid extraction kits, should perform according to specifications. Modifications to manufacturer's instructions should be validated and documented. The presence of inhibitors and interfering substance that can interfere with downstream analyses should be assessed.

4.1.3 There should be a documented process for validating in-house methods

4.1.3.1 In-house methods should be validated in ways commensurate with the intended clinical purpose of the test. Validation parameters may include:

a) comparison with other existing molecular and conventional laboratory tests
b) use of control or reference material
c) test for inhibitors and contamination
d) limit of detection and dynamic range
e) sensitivity (with reference to a second method or clinical studies)
f) specificity
g) precision and reproducibility
h) correlation with clinical diagnosis

4.1.3.2 A written procedure giving adequate and precise details on the reagents (source, preparation, storage, and stability) and methods used must be available for each test.

a) Adequate information about any primer(s) or probe(s) used in any assay must be recorded to permit interpretation and troubleshooting of results.

b) For tests that generate a result based on a melting temperature (Tm), appropriately narrow temperature ranges (+/- 2.5ºC) are defined and recorded each day of use

c) For gel based assays, there should be written criteria for band resolution and gel quality. Known molecular weight markers that span the range of expected bands should be used for each electrophoretic run. Visual or fluorescent markers are used to determine the endpoint of gel electrophoresis.

4.1.3.3 In laboratory-developed tests for pathogen detection, the chosen target sequence should be checked against a databank for homology to the desired pathogen and to ensure lack of cross-reactivity to other closely related organisms, prior to adoption of the method.

4.1.3.4 Limit of detection may be determined using dilution series of artificial construct or characterized clinical specimen

4.1.3.5 Assay (Analytical) specificity should be evaluated (using ≥ 30 samples) against a reference method and/or using known samples.

The threshold for differentiating a positive and negative test should be determined. This may also take into account assays with multiple targets. Criteria and terminology for an equivocal range, if needed, should be clearly defined. Appropriate interpretive comment should be included.

4.1.3.6 The assay should be tested for cross-reactivity against at least 30 samples containing commensal or potentially cross-reacting organisms when the samples are available. Whenever possible, these samples should include all relevant species and types. In such event when the samples are not available, the laboratory should demonstrate the specificity or cross-reactivity by testing against sample type-specify genomic background and comparing genomic sequences of potentially cross-reacting organisms using bioinformatics tools while the laboratory continues to source and collect such samples.

4.1.3.7 If the assay specificity is sub-optimal, a confirmatory test targeting a different region of the pathogen genome should be used.

4.1.3.8 Assay (Analytical) sensitivity should be tested (using ≥ 30 samples) against a reference method, known positive specimens, or spiked specimens. For disease of low occurrence rates, at least 20 samples should be tested.

4.1.3.9 Dynamic range of quantitative assays: The lower and upper limit of quantification in reportable units e.g. IU/ml or copies/ml, should be determined using known standards.

4.1.3.10 Linearity range of quantitative assays: The assay linearity should be established using at least 4 replicates for each dilution.

4.1.3.11 Assay precision and reproducibility: Between-batch (day) variation should be evaluated using at least two dilutions near the cut-off limit and tested in 20 individual runs.

4.1.3.12 For assays relying on presumptive identification based on a single band on a gel, or on melting point for real-time PCR, the identity of the products of at least 20 samples should be checked during validation by sequencing, probe recognition or other methods.

4.1.3.13 The acceptable range of amplified product size or melting point that calculated by analytical instruments should be established by testing at least 30 samples.

4.1.3.14 For each step of the procedure all incubation temperatures are defined and recorded. Incubations (reactions) performed using baths/blocks/instruments meet manufacturer's specifications.

4.2 Sequencing

4.2.1 There shall be written criteria for the acceptability and interpretation of primary sequencing data.

4.2.2 The laboratory shall have a procedure in place to ensure that interpretation of sequence data is based on the latest version of the manufacturer's interpretive software.

4.2.3 The laboratory shall have a policy in place to assure that appropriate databases (that are comprehensive and current) are used for the interpretation of sequencing data.

4.2.4 If the laboratory uses alternative sequence interpretive databases, either alone or in conjunction with manufacturer's software, the alternative databases have been validated for the interpretation of the sequence data.

4.2.5 Sequence data are to be correlated with available phenotypic data.

4.2.6
a) If Sanger sequencing is the methodology of an analysis or used to confirm results, the validation and written protocols may also include the following: Calibration and re-calibration schedule of sequencer and capillaries;
b) Shelf life and validity of capillaries and all sequencing reagents;
c) Selection of capillary and polymer;
d) Primer selection and design;
e) Amplification parameters including template concentration, formulation of amplification reaction and PCR cycling conditions;
f) PCR product purification;
g) Acceptable length of reads and quality score;
h) Software selection of base calling, sequence alignment and review;
i) Criteria for variant calling;
j) Criteria for manual sequence editing;
k) Trouble shooting strategy and procedure of low quality runs;
l) Selection of reference sequences;
m) Algorithm and parameters of BLAST, sequence alignment and phylogenetic tree if applicable;
n) Retention period and backup schedule of different sequence files

4.3 Next Generation Sequencing:

In-house bioinformatics pipelines should be validated. Any revision or change of software/pipeline version must be documented for commercial or –laboratory developed pipelines.

4.4 Post-validation monitoring

4.4.1 This should be done for both in-house and commercial methods, and includes review of clinical cases and updated published reports in the scientific literature. A logbook may be kept for unusual or instructive cases.

4.4.2 An annual review should be done to investigate possible cross-reactivity of amplified target sequence to newly published sequences deposited in gene databanks.

4.5 Storage of Nucleic Acids

4.5.1 Nucleic acids should be processed promptly and stored adequately to minimize degradation.

Isolated nucleic acid should be stored in tightly capped containers, for which both the containers and caps should be labelled by sample unique identifiers. Long-term storage should be carried out at -20° or -70°C to prevent degradation. Stability of the sample can be maintained for several months by storing at 4°C. Integrity of samples should be re-evaluated before use if stored for prolonged periods of time at any temperature.

5. Ensuring the Validity of Examination Results

5.1 Quality Control

5.1.1 For electrophoretic separations, known molecular weight markers should be used for each run and they should span the range of the expected bands.

5.1.2 Positive (including low positive) and negative controls should be run for each assay, when available, appropriate and practical. For panels with multiple targets, systematic rotation of controls may be acceptable.

5.1.3 False negatives should be excluded. This may be done by one or more of the following:

- using an inhibitor control with each run
- establishing the false-negative rate during the analytical validation phase, and during continual monitoring of test result trends
- establishing the suitability of specimen type for the particular test in prior studies
- using reagents tested for inhibitory properties for the specimen type
- referring to published literature using the same reaction conditions

5.1.4 The use of an internal inhibitor control in each run should be determined on a case-by-case basis, depending on the following factors:

a) likelihood of encountering inhibitors e.g. crude or contaminated clinical specimens like sputum or stool
b) performance (sensitivity, specificity, precision) related to conventional methods

c) clinical implications of a false-negative result
d) the degree to which the clinical diagnosis rests on the result of the laboratory procedure
e) available data on inhibition using the specific specimen type

5.1.5 For commercial kits approved as in vitro diagnostic devices by the regulatory authorities, it is sufficient to follow the manufacturer's instructions and use the manufacturer's controls.

When manufacturer's controls are not available, e.g. commercial nucleic acid extraction kits, the laboratory should have a written procedure to perform quality control for each batch of the kits.

5.1.6 For quantitative assays, quality control statistics are calculated monthly to define analytic imprecision and to monitor trends over time.

5.1.7 For multiplex tests, at least two analytes are individually verified for each new shipment and lot, and the analytes verified are periodically rotated.

5.1.8 For thermocyclers, individual wells (or a representative sample thereof) are checked for temperature accuracy before being placed in service and at least annually thereafter.

5.1.9 When external service laboratory or company is engaged in any step of testing or result confirmation, such as sequencing confirmation, the laboratory should ensure the laboratory complies with ISO 15189 or meet specified quality criteria. QC files should be recorded for each run. The laboratory may conduct inspection and review the protocols regularly and send in known samples as blind test to monitor the service quality.

5.2 <u>External quality assessment</u>

5.2.1 The laboratory shall participate in external quality assessment (EQA) for the tests offered. Where EQA for an analyte is not available, performance assessment must be conducted at suitable intervals (e.g., six-monthly) by appropriate procedures. These procedures include: split sample analysis with other laboratories, split samples with an established in-house method, assayed and reference materials, clinical validation by chart review, or other suitable and documented means.

5.2.2 Discrepancies between the molecular pathology laboratory's results, other laboratory findings, and the clinical presentation should be investigated and documented, along with any corrective action.

6. Reporting of Results

6.1 There should be a policy on criteria for acceptable results that may be released and criteria for doubtful results that should be either be repeated or require additional investigation.

6.2 There should be a procedure for defining critical results and notification of such results.

6.3 There should be a procedure for issue of amended reports following issue of erroneous results. Laboratory errors should be investigated, immediate and preventive actions implemented and documented.

6.4 The final report should include sufficient information about the method used, and for certain tests, subjective or interpretative comments, including references where relevant. For in-house tests the method used must be documented. The report should be formatted in such a way as to allow easy and correct clinical interpretation.