

NATIONAL PATHOLOGY ACCREDITATION ADVISORY COUNCIL

**REQUIREMENTS FOR MEDICAL
TESTING OF MICROBIAL NUCLEIC
ACIDS**

(Second Edition 2013)

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Second edition 2013 reprinted and reformatted to be read in conjunction with the *Requirements for Medical Pathology Services*

Australian Government Department of Health

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The National Pathology Accreditation Advisory Council (NPAAC) was established in 1979 to consider and make recommendations to the Australian, state and territory governments on matters related to the accreditation of pathology laboratories and the introduction and maintenance of uniform standards of practice in pathology laboratories throughout Australia. A function of NPAAC is to formulate Standards and initiate and promote education programs about pathology tests.

Publications produced by NPAAC are issued as accreditation material to provide guidance to laboratories and accrediting agencies about minimum Standards considered acceptable for good laboratory practice.

Failure to meet these minimum Standards may pose a risk to public health and patient safety.

Scope

The *Requirements for Medical Testing of Microbial Nucleic Acids* is a Tier 4 NPAAC document and must be read in conjunction with the Tier 2 document *Requirements for Medical Pathology Services*. The latter is the overarching document broadly outlining standards for good medical pathology practice where the primary consideration is patient welfare, and where the needs and expectations of patients, Laboratory staff and referrers (both for pathology requests and inter-Laboratory referrals) are safely and satisfactorily met in a timely manner.

Whilst there must be adherence to all the Requirements in the Tier 2 document, reference to specific Standards in that document are provided for assistance under the headings in this document.

The Standards and Commentaries set out in this document are intended to apply to tests used for the detection, characterisation and quantification of nucleic acids (deoxyribonucleic acid or DNA and ribonucleic acid or RNA) in medical Laboratories testing for microorganisms that can or may cause disease in humans.

This document has been promulgated for use in the accreditation of Laboratories in the above medical context only.

This document does not address issues relating to non-human Specimens (e.g. food testing), instrument testing, testing for infectious proteins (i.e. prions), environmental sampling, point of care testing and new technologies currently not in routine use in diagnostic Laboratories.

Abbreviations

AS	Australian Standard
ASM	The Australian Society for Microbiology Inc.
bDNA	Branched-Chain DNA
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HPV	Human Papilloma Virus
ISO	International Organization for Standardization
IVD	In Vitro Diagnostic Devices
LIMS	Laboratory Information Management System
mRNA	Messenger RNA
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
NASBA	Nucleic Acid Sequence Based Amplification
NAT	Nucleic Acid Testing
NHMRC	National Health and Medical Research Council
NPAAC	National Pathology Accreditation Advisory Council
OGTR	Office of the Gene Technology Regulator
PCR	Polymerase Chain Reaction
PPM	Parts Per Million
TMA	Transcription Mediated Amplification

Definitions

Confirmatory testing	<p>means:</p> <ol style="list-style-type: none"> 1. a procedure performed to verify the truth or validity of something thought to be true or valid. 2. testing performed to assure that a result achieved is the correct result or is the final test performed to achieve the true diagnosis.
Contained area	means an area that can be physically isolated from other areas either by a physical barrier or within the working space of an instrument.
Contamination	means where the unintentional introduction of nucleic acids from a source, other than the intended Specimen being tested, into the assay can or has the potential to influence the interpretation of the assay. Contamination can be either cross-contamination or carry-over contamination.
Cross-contamination	means where nucleic acid from another Specimen or operator(s) is the source of the contamination. These nucleic acids have not been produced in vitro.
Carry-over contamination	means where the nucleic acid from an in vitro assay such as a PCR amplicon is the source of the contamination.
Nucleic acid test (NAT)	means a testing assay for the detection and/or characterisation of DNA and RNA and include those that involve target amplification (PCR), signal amplification, branch chain DNA (bDNA) assay, DNA sequencing etc.
Requirements for Medical Pathology Services (RMPS)	<p>means the overarching document broadly outlining standards for good medical pathology practice where the primary consideration is patient welfare, and where the needs and expectations of patients, Laboratory staff and referrers (both for pathology requests and inter-Laboratory referrals) are safely and satisfactorily met in a timely manner.</p> <p>The standard headings are set out below –</p> <p>Standard 1 – Ethical Practice Standard 2 – Governance Standard 3 – Quality Management Standard 4 – Personnel Standard 5 – Facilities and Equipment A – Premises B – Equipment Standard 6 – Request-Test-Report Cycle A – Pre-Analytical B – Analytical C – Post-Analytical Standard 7 – Quality Assurance</p>

Separate area	means an area that is functionally separated from other areas by physical barriers, distance or strictly defined practice, or by performance of the test within the working space of an instrument, as dictated by the methods and technology available in the Laboratory.
Validation* ISO9000:2005 3.8.4	means the confirmation by examination and the possession of objective evidence that the particular requirements for a specific intended use are fulfilled.
Verification* ISO9000:2005 3.8.5	means the application of the validation process only to a nonconforming aspect of an otherwise validated IVD. Verification can comprise activities such as: <ul style="list-style-type: none"> • performing alternative calculations • comparing a new design specification with a similar design specification • undertaking tests and demonstrations • reviewing documentation prior to issue.

* Definition from ISO/FDIS-WD 14971:2005, *Medical Devices – Application of Risk Management to Medical Devices*

Introduction

Laboratory testing for microbial pathogens including viruses, bacteria, fungi and parasites has changed significantly with the availability of nucleic acid testing (NAT) assays. The most significant changes have been in the range of techniques available – in addition to polymerase chain reaction (PCR), there are many other methods now available. Further characterisation of microbial pathogens using sequencing of target genes, determination of resistance genotypes by sequencing key enzymes and genotyping are all now routinely available for a small number of infectious agents. In many cases, NAT assays have replaced routine testing (for example, in diagnosis of many viral infections). NAT assays often yield increased sensitivity, specificity and information relating to the pathogen. The counter to this has been additional need for testing facilities, particularly to reduce the risk of contamination due to the sensitive nature of NAT assays.

The *Requirements for Medical Testing of Microbial Nucleic Acids* set out the minimum Standards for using NAT assays to diagnose and monitor infection. The document is directed towards Laboratories using NAT assays in medical diagnosis.

Human genetic testing is addressed in the *NPAAC Requirements for Medical Testing of Human Nucleic Acids*.

These Requirements are intended to serve as minimum Standards in the accreditation process and have been developed with reference to current and proposed Australian regulations and other standards from the International Organization for Standardization including:

AS ISO 15189 *Medical laboratories – Requirements for quality and competence*

These Requirements should be read within the national pathology accreditation framework including the current versions of the following NPAAC documents:

Tier 2 Document

- *Requirements for Medical Pathology Services*

All Tier 3 Documents

Tier 4 Document

- *Requirements for Laboratory Testing for Antibodies to the Humans Immunodeficiency Virus (HIV) and the Hepatitis C Virus (HCV)*

In each section of this document, points deemed important for practice are identified as either ‘Standards’ or ‘Commentaries’.

- A Standard is the minimum requirement for a procedure, method, staffing resource or facility that is required before a Laboratory can attain accreditation – Standards are printed in bold type and prefaced with an ‘S’ (e.g. **S2.2**). The use of the word ‘**must**’ in each Standard within this document indicates a mandatory requirement for pathology practice.

- A Commentary is provided to give clarification to the Standards as well as to provide examples and guidance on interpretation. Commentaries are prefaced with a ‘C’ (e.g. C1.2) and are placed where they add the most value. Commentaries may be normative or informative depending on both the content and the context of whether they are associated with a Standard or not. Note that when comments are expanding on a Standard or referring to other legislation, they assume the same status and importance as the Standards to which they are attached. Where a Commentary contains the word ‘**must**’ then that Commentary is considered to be **normative**.

Please note that the Appendices attached to this standard are **informative** and should be considered to be an integral part of this document.

In addition to these Standards, Laboratories must comply with all relevant state and territory legislation (including any reporting requirements).

Please note that all NPAAC documents can be accessed at www.health.gov.au/internet/main/publishing.nsf/Content/health-npaac-publication.htm

While this document is for use in the accreditation process, comments from users would be appreciated and can be directed to:

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1. Pre-analytical phase

(Refer to Standard 1, Standard 4 and Standard 6A in *Requirements for Medical Pathology Services*)

S1.1 The Laboratory must comply with the guidelines of the OGTR when using recombinant nucleic acids.

2. Specimen suitability and collection

(Refer to Standard 6 in *Requirements for Medical Pathology Services*)

Care needs to be taken to ensure that DNA and RNA remain intact during Specimen storage, transport and preparation. If the number of target sequences in the Specimen is very small, a false negative result may be obtained if degradation occurs.

S2.1 If patient-collected Specimens are to be used for diagnosis, clear instructions must be provided to the patient, to reduce the likelihood of Specimen contamination and maintain Specimen integrity.

S2.2 NAT assays must be performed on dedicated Specimens or dedicated aliquots. If clinical necessity dictates that a non-dedicated Specimen must be used, then this must be recorded by the Laboratory on the report.

C2.2(i) Special Specimen collection and preparation is needed, in addition to usual requirements for pathology testing in order to minimise the risk of contamination in NAT assays.

C2.2(ii) The precise method of Specimen collection, initial processing and transportation depend on the Specimen concerned and the nucleic acid target (DNA or RNA).

3. Laboratory facilities

(Refer to Standard 5 in *Requirements for Medical Pathology Services*)

Laboratories undertaking NAT assays should be configured to minimise the risk of contamination of Specimens and reagents by other Specimens in the Laboratory or by amplified material.

In microbiology Laboratories, microorganisms are present in Specimens in large numbers or are cultured at high concentrations. There is a potential for aerosol contamination because of the small size of most microorganisms.

The wording of the following sections is intended to allow flexibility of layout without compromising the guiding principle that Laboratories undertaking nucleic acid testing should be configured to minimise the risk of contamination.

Minimum standards for a NAT assay facility using target amplification

S3.1 At least three contained areas must be provided in order to reduce the risk of cross-contamination and carry-over contamination. The normal airflow pattern between each of the areas and the layout of the Laboratory must be designed to minimise the potential for aerosol cross-contamination. The three contained areas required are for:

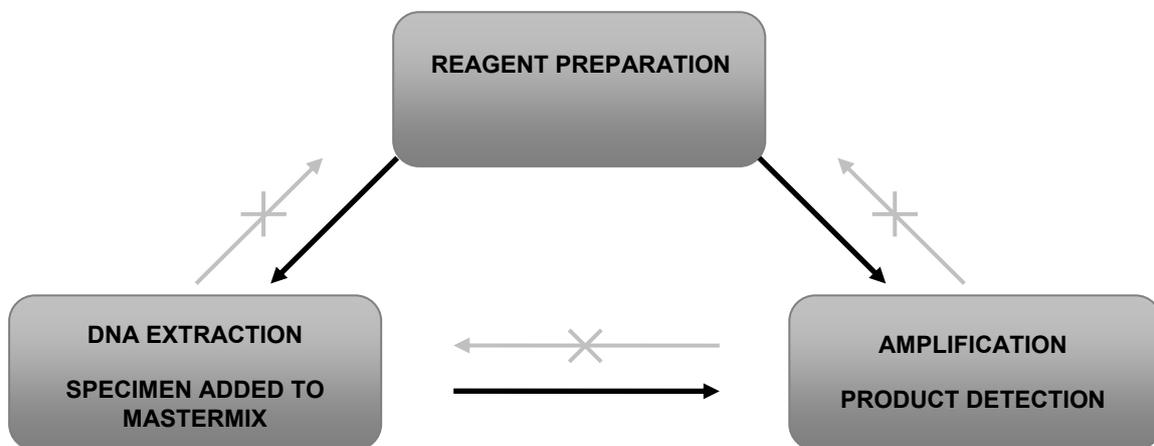
- (a) the preparation of reagents**
- (b) the extraction of nucleic acids from Specimen and for the addition of Specimen DNA to tubes containing master mix before PCR amplification**
- (c) amplification and product detection.**

C3.1(i) There **must** be attention to room cleanliness and regular surface cleaning with an appropriate agent.

C3.1(ii) Work surfaces **must** be regularly decontaminated with a suitable decontaminating agent. Instrumentation such as microcentrifuges, heating blocks and water baths **must** be cleaned regularly with a suitable, non-corroding decontaminating agent where appropriate.

C 3.1 (iii) The time intervals for cleaning and decontaminating equipment and areas **must** be documented.

- C3.1(iv) Schematic representation of areas required to minimise cross-contamination. Separate areas may be separate rooms or contained areas such as within an instrument.



- C3.1(v) Instruments capable of producing aerosols such as vortex mixers, PCR machines and centrifuges should be placed at as great a distance from preparation areas as possible.
- C3.1(vi) The use of robotic equipment in the Laboratory for NAT assays should adhere to the Standards outlined herein.

S3.2 If material from the first-round PCR is to be subjected to further amplification, such as in a nested PCR, these materials must be handled in a fourth separate area.

- C3.2(i) These manipulations of Specimens or of materials liable to contain amplified or other nucleic acids can be carried out in a Class I Biological Safety Cabinet (BSC), a Class II BSC with a High-Efficiency Particulate Absorbing (HEPA) filter on the exhaust, or within an instrument.
- C3.2(ii) Equipment designated for a particular area should be marked (e.g. by colour) to clearly indicate to which area it belongs.

S3.3 Movement of reagent, Specimens, equipment and personnel between separate areas must be minimised.

- C3.3(i) Reagents **must** be limited to the appropriate areas. Amplified nucleic acid Specimens **must not** be taken into the reagent preparation area. Specimens and amplicons **must** be stored separately from reagents.

- C3.3(ii) The movement of Specimens **must** be unidirectional, that is, from pre-amplification to post-amplification areas. Only sealed PCR amplification tubes and tube racks are to be carried between the pre-amplification area and the post- amplification area.
- C3.3(iii) Dedicated equipment **must** be available in each area. If equipment is returned against the Specimen flow, it **must** first be decontaminated using a suitable agent.
- C3.3(iv) Laboratory coats and gloves **must** be changed before staff move between areas where there is risk of contamination. Where shoe coverings are used in the reagent preparation room, these **must** also be changed before moving to other areas.
- C3.3(v) Aerosol-resistant pipette tips or positive displacement pipettes are strongly recommended to minimise contamination, and should be used routinely.

S3.4 Normal airflow patterns in the Laboratory must be designed to minimise cross-contamination between the three areas previously described in S3.1.

- C3.4(i) The greatest hazard arises from amplicons but care should also be taken with extracted nucleic acids.
- C3.4(ii) Because of the nature of the technique, nested PCR requires extreme care. The most important aspects are:
 - (a) use of aerosol-resistant pipette tips
 - (b) constant vigilance against methodological causes of contamination
 - (c) other methods of nucleic acid decontamination such as UV radiation.

4. Analytical phase

(Refer to Standard 6B in *Requirements for Medical Pathology Services*)

Testing for infectious pathogens of humans using NAT assays falls into detection of DNA and RNA. However, in clinical practice the major division is between detection of fungi (which often have complex coats making processing slightly more difficult to extract DNA), bacteria (containing DNA and RNA) and viruses (containing DNA or RNA but not both). Importantly, although techniques overlap between these three classes of organisms (eukaryotic, prokaryotic and viruses), the techniques are often altered to take account of the differential nucleic acid, protein and other content of the organism.

5. Validation and verification of NAT assays

(Refer to Standard 7 in *Requirements for Medical Pathology Services*)

Procedures for the validation and verification of qualitative and quantitative NAT assays can be found in the NPAAC Tier 3 document *Requirements for In-House In Vitro Diagnostic Devices*.

S5.1 All assay runs must include positive and negative controls that are subject to the whole test process, including the extraction.

- C5.1(i) Where a negative result is obtained, there **must** be confirmation that nucleic acid has been extracted, that is, a positive control and internal control must be included.
- C5.1(ii) In assays using RNA as the starting material, controls **must** be incorporated to detect the success of RNA extraction and of reverse transcription.
- C5.1(iii) Where it is not feasible to test all positive controls in a single batch or run, all controls **must** be tested over a cycle and this must be documented.
- C5.1(iv) Positive controls may consist of patient Specimens containing target nucleic acid or negative Specimens spiked with whole organism or purified nucleic acid.
- C5.1(v) Where test runs are expected to contain a large proportion of positive results, additional ‘no nucleic acid’ controls should be interspersed among the patient Specimens at an appropriate frequency, as validated by the particular Laboratory.

6. Characterisation including genotyping and resistance testing

(Refer Standard 6 in *Requirements for Medical Pathology Services*)

Microorganism characterisation has undergone continual refinement as a consequence of the development of new technologies. Genetic sequences within species can be generally subdivided into groups, commonly termed genogroups, clades or genotypes. Once sequence information has been generated, phylogenetic analysis may be used to establish relationships between organisms, group clusters of related sequences and to estimate rates of evolution.

While there may be no observable phenotypic differences between the genotypes of some microorganisms, for others, genotyping may correlate with disease pathogenesis, infectivity and clonality for infection control purposes as well as response to antimicrobial agents.

S6.1 The genotyping method must be sufficiently discriminatory to allow useful distinction between relevant isolates.

C6.1 Genotyping may be used for individual patient care, infection control purposes, tracing of clonal microorganisms, and epidemiological investigations.

S6.2 When using DNA sequencing, the Laboratory must confirm that the sequencing methodology is appropriate, for example, Basic Local Alignment Search Tool (BLAST) search or comparison with a known standard sequence. The Laboratory must establish the standard of quality for the acceptance of sequence data.

C6.2(i) Software is used to interpret genotypic results for some characterisation tests, for example, virtual resistance phenotypes established from genotypic data. Such software should be assessed by the Laboratory as fit for purpose, accurate, and considered as part of the validation for an IVD.

C6.2(ii) In order to prevent mis-identifications in sequencing, procedures should include order of Specimens for sequencing and positioning of identifiable controls.

C6.2(iii) The reasons a Laboratory may wish to sequence a PCR product may include:

- (a) mutation screening, which is the detection of an unknown mutation in a length of DNA or RNA
- (b) confirmatory testing
- (c) genotyping
- (d) microorganism identification.

7. Conflicting tests and possible contamination

(Refer Standard 6 in *Requirements for Medical Pathology Services*)

Contamination is the most frequent adverse issue related to NAT assays in microbiology testing. This is a function of the high sensitivity of the assay, the use of target amplification techniques, the large number of amplicons generated in a given assay that potentially can contaminate surfaces within the Laboratory, and other aspects of the NAT assays. There are procedures in place in the analytical phase of NAT assays to minimise and detect contamination. Reports may be issued which, in light of subsequent information, are incorrect due to undetected contamination. Such information might include additional NAT assays, or supplemental assays on the same patient.

Result amendment due to contamination is not confined to tests for microorganisms, but is an issue for NAT assays generally.

S7.1 All Laboratories must be aware of the risk of contamination within NAT assays and have documented procedures for reviewing of suspected false positive and false negative results.

C7.1 It is strongly recommended that where the implications of a positive result are substantial (for example, sexually transmitted diseases) and where the specificity of the test is suboptimal, one or more of the following methods be used to verify positive results:

- (a) repeat testing of all positives, this may include using another set of primers directed at a different target sequence
- (b) nested PCR or other techniques that require the binding of more than one set of primers to generate a positive result
- (c) identification of the product using methods such as specific probes, sequencing or restriction enzyme analysis.

S7.2 Laboratories must retain records documenting contamination events, which must include the identified source of the contamination if known and measures taken to reduce the risk of future similar contamination events.

Supplemental and additional testing

Laboratories may use supplemental testing in selected situations. These supplemental tests may be reported alongside NAT assays. Such supplemental assays may include separate NAT assays or alternative tests. There are many NAT assays where the test itself is the most sensitive test available.

Any single nucleic acid amplification test may be part of a testing algorithm that uses supplemental testing to improve specificity. A similar method is used in serological testing in which a sensitive but non-specific screening assay is followed by supplemental tests (e.g. the western blot for human immunodeficiency virus) to improve the specificity of the result.

S7.3 Laboratories must have available access either on-site or off-site to at least one supplemental test for NAT assays.

C7.3(i) Supplemental tests for NAT include:

- (a) serology – seroconversion to IgG, IgM tests, antibody avidity tests, and quantitative antibody assays
- (b) culture of the microorganism
- (c) detection of antigens of the organism, for example, using fluorescence or enzyme immunoassays
- (d) an alternative NAT assay.

C7.3(ii) In many cases supplemental testing will be less sensitive than NAT assays. The Laboratory should have an alternate assay available where possible.

C7.3(iii) Laboratories performing NAT assays should confirm test specificity and be aware that false positive results may occur for reasons other than contamination. These may occur through:

- (a) non-specific primer binding and amplification of other sequences, which are then misidentified as the target sequence
- (b) amplification of similar or identical target sequences found in other non-target-containing genomes
- (c) non-specific, usually low-level, reactivity in the detection system.

C7.3(iv) Laboratories performing NAT assays should confirm test sensitivity and be aware that false negative results may occur through:

- (a) changes in the target sequence
- (b) limitations of the methodologies of detection.

C7.3(v) The frequency and significance of these events will vary with the test technique, the target sequence chosen, the detection system used and the patient population.

S7.4 Laboratories must be able to determine when supplemental results require the revision of the original report and the issuing of an amended report.

S7.5 The Laboratory must have in place procedures for providing amended reports where the supplemental and original tests conflict.

8. Post-analytical phase

(Refer to Standard 6C in *Requirements for Medical Pathology Services*)

Reporting of results

A particular challenge in reporting NAT assays is that knowledge of microorganism and genomics testing methodology is changing rapidly, with consequent changes in test performance and interpretation. There is great diversity in the clinical context, complexity, and significance of NAT assay use, and no single report format would be able to address this wide range of requirements. Apart from the performance of a NAT assay, the interpretation of the result is critical. The Laboratory should ensure that the way in which the result is given facilitates its interpretation.

S8.1 The methodology used for detection of nucleic acid (for example, PCR) must be included in the report of a NAT assay.

- C8.1(i) Reports should indicate the genotypes detected by a given assay, where the genotypes are known and relevant.
- C8.1(ii) The Laboratory should use standard taxonomy for referring to Specimens in reports.

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Appendix A Template for Molecular testing validation for an in-house assay (Informative)

Whilst validation must be performed in accordance with the NPAAC *Requirements for the Development and Use of In-house in-Vitro Diagnostic Devices (IVDs)*, below is a template that may be useful for the technical aspects of validation.

Assay validation will be performed in two major steps:

1) Developmental Validation: this addresses specificity, sensitivity, accuracy, bias, precision, false positives and false negatives. Appropriate controls should be determined and reference databases used should be documented.

2) Operational Validation: once a method has been developed and initially validated, it may be transferred to the Laboratory as an operational method for implementation. Operational validation is accumulation of test data during testing to demonstrate that the established method(s) and procedure(s) are carried out within predetermined limits in that Laboratory. This includes quality control, competency assessment of personnel, proficiency testing and instrument calibration.

1. Developmental Validation

1.1 Description of Test

Note: Appropriate personal protective equipment (PPE) and environmental containment conditions must be used when working with infectious organisms.

1.2 Test Method Protocol

1.2.1 Primers and Probes:

Primers and Probe	Sequence	Working Concentration	Reference

1.2.2 Mastermix:

All reagents must be stored and used according to the manufacturer's instructions.

Component	Lot No	Concentration	Volume / reaction	Vol / working run
Reaction mix				
Forward primer				
Reverse primer				
Probe				
Enzyme				
Deionised water				
Template				
Other				

1.2.3 Dilutions: *Outline and list any dilutions of probes, primer or other reagents*

1.2.4 Describe Set-up: *Outline steps and sequence of actions to set up reactions*

1.2.5 Describe Cycling Conditions: *Identify thermocycler instrument and describe conditions and list saved run name if appropriate*

1.2.6 Description of Detection Method and Acceptance Criteria:

List criteria which must be fulfilled for the run to be acceptable

Controls	Acceptable Condition
No template control	No amplification
Primer and probe controls	Amplification within accepted limits
Internal cycling control	Amplification within accepted limits
Positive control for template	Amplification within accepted limits

1.3 BLAST analysis of primers and probes

Sequence	Acceptable
Forward primer unique to target	Yes / No
Reverse primer unique to target	Yes / No
Probe sequence unique to target	Yes / No

Note: If probes or primers not unique to target give explanation.

1.4 Evidence of clinical or biological association

Some infectious diseases are defined qualitatively i.e. the presence of an organism at any site e.g. *Mycobacterium tuberculosis* or the detection of an organism in an otherwise sterile site e.g. *Neisseria meningitidis* in cerebrospinal fluid is indicative of infection.

Some infectious diseases are defined quantitatively e.g. cytomegalovirus or Epstein Barr virus as copies per unit of substrate.

Note: It is often difficult to determine if the detection of an organism is indicative of diseases as both live and dead (non-replicating) organisms are detected using molecular methods. Test results must be assessed within the clinical and epidemiological context.

1.5 Reagents and Consumables

All reagents and consumables must be obtained from approved suppliers and a record kept.

All reagents and consumables must have Lot No and expiry date recorded.

All reagents and consumables must have passed internal or external quality control and the result recorded.

All reagents and consumables must be stored under appropriate environmental conditions. Records of environmental conditions must be kept.

1.6 Equipment:

All equipment used must be under calibration controls where appropriate and records kept.

All equipment used must be under maintenance controls and records kept.

1.7 Optimisation

Document any further optimisation of the test procedure.

1.8 Limits of Detection

Detail any procedure and results where the limits of detection of the assay were determined e.g. dilution of substrate containing target, no of bacterial colonies, quantity of virus, and number of transcripts using a plasmid containing the template target.

1.9 Precision

The guideline for precision (reproducibility) of a qualitative assay (positive or negative) result is determined by testing the positive and negative control material for 20 runs or in duplicate for 10 runs. If any control does not perform as expected the run must be rejected. If there are more than 1 in 10 run failures over a period of time corrective action is required.

The guideline for precision (reproducibility) of a quantitative assay requires the determination of the acceptable upper and lower limits of detection including confidence intervals using multiple separate test runs. In addition intra-assay co-efficient of variation (CV) should be determined.

Suggest: Data from limits of detection used in replicate runs be used to determine the lower and upper limits of detection including confidence limits e.g. Ct from real-time instrument.

1.10 Accuracy

Accuracy is the ability of the test to detect the correct result. The accuracy of a new test system is evaluated by testing archived positive and negative Specimens, positive control material, and culture material in parallel with a comparative method.

Suggested: Quantitative assays require both comparative and new test method together for a minimum of 20 duplicate Specimens over a number of runs over a minimum of 5 days. The concentration of analyte should cover low, medium and high within the range of detection.

1.11 Bias

Bias is the systematic analytical deviation from the true value and is either positive or negative.

Suggested: When a quantitative assay is performed the bias should be determined. The total error of the assay is the sum of imprecision (coefficient of variation of the assay) and bias.

1.12 Linearity

The linearity of a quantitative assay must be established for the relevant clinical range.

Suggested: Test a dilution series of not less than 5 dilutions of a known strong positive whose genome level has been compared with a known genome copy number. Each dilution should be test four times in the same assay run and the results plotted against the nominal copy number.

1.13 Sensitivity

Diagnostic tests are never perfect. False positive and false negative results occur. How much of a problem these false results may cause depends on the clinical context in which a test is used.

Selecting the optimal balance of sensitivity and specificity depends on the purpose for which the test is going to be used. A screening test should be highly sensitive and a confirmatory test should be highly specific.

Sensitivity: the ability of a test to detect a true positive. Specimens known to be positive in a “gold standard” assay or from individuals who exhibit specific symptoms and signs of the disease should be tested and the assays sensitivity determined using the following formula.

$$\text{Sensitivity} = [\text{True Positive} / (\text{True Positive} + \text{False Negative})] \times 100\%$$

Where relevant, all serotypes or genotypes of the organism being tested should be included in the assay. The suitability of the assay must be demonstrated with all Specimen (substrate) types.

1.14 Specificity

Specificity: the ability of a test to detect a true negative. Specimens of organisms with similar taxonomy, found in the same clinical Specimen or producing a similar clinical disease should be tested and the assays specificity determined using the following formula.

$$\text{Specificity} = [\text{True Negative} / (\text{True Negative} + \text{False Positive})] \times 100\%$$

Where relevant, all serotypes or genotypes of the organism being tested should be included in the assay. Testing of 100 Specimens that do not contain analyte should be tested to confirm specificity.

1.15 Interfering Substances

It is essential to determine the effect of potential interfering substances on assay performance.

Suggested: The effect of interfering substances will be determined by the testing for the target in all substrates relevant for the assay in question. This would include but is not limited to urine, blood, sputum, nasopharyngeal aspirates, cerebrospinal fluid, joint fluid, pleural fluid, various tissue types, faeces with an internal control.

1.16 Training

It is essential to ensure that any staff member who performs test has appropriate qualifications and training. A training program specific for the test may need development. All training should be documented and records kept.

1.17 Result Reporting

The test results must be reported in a manner that is understandable by the clinician who ordered the test with appropriate interpretative comments and reference ranges when required. The test report must include all required information.

2. Internal validation

The assay should be reviewed regularly to determine clinical relevance and the relevance of the technical approach in the light of new information.

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Appendix B Verification of a commercial NAT assay (Informative)

This summarises the type of verification a Laboratory may undertake.

Rapid Direct Detection of MRSA Nasal Colonisation

Several commercial rapid, qualitative, multiplex PCR-based IVDs for detecting MRSA directly from nasal swab Specimens are available in Australia. These assays determine the presence of MRSA through real-time PCR amplification and fluorogenic target-specific hybridisation.

Several published studies have shown these assays have a high negative predictive value (>99%). However genetic variability within the assays' targeted region (junction region of the Staphylococcal Cassette Chromosome *mec* [SCC*mec*]) has been demonstrated and consequently false negative results occur particularly with those strains harbouring, although not limited to SCC*mec* types V, VII and VIII. Consideration should therefore be given to verifying the assay using locally acquired MRSA strains. The Australian Group for Antimicrobial Resistance (<http://www.antimicrobial-resistance.com/>) can provide, on request, a panel of fully characterised community-associated and healthcare-associated MRSA strains collected within a particular region of Australia as part of its annual surveillance programmes. All enquiries should be directed to info@agargroup.org. MRSA strains within a region may vary over time, and therefore Laboratories should consider regularly verifying the assay against local isolates.

Laboratories should also be aware that these assays may have a very low positive predictive value (<70%). This may be due to the presence of non-viable organisms or due to *S aureus* isolates with an inactivated *mecA* gene or with genetic elements similar in structure to the SCC*mec* but encoding functions unrelated to methicillin resistance.

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Bibliography

1. Attanoos RL, Bull AD, Douglas-Jones AG, Fligelstone LJ, Semarano D (1996). Phraseology in pathology reports. A comparative study of interpretation among pathologists and surgeons. *J Clin Pathol* 49:79-81.
2. *Collection, Transport, Preparation and Storage of Specimens for Molecular Methods; Approved Guidelines*. CLSI, 2008.
3. Fredericks DN and Relman DA (1996). Sequence-based identification of microbial pathogens: a reconsideration of Koch's postulates. *Clinical Microbiology Reviews* 9:18–33.
4. *Genotyping for Infectious Diseases: Identification and Characterization; Approved Guideline*. CLSI, 2008
5. Gulley ML, Braziel RM, Halling KC, Hsi ED, Kant JA, Nikiforova MN et al (2007). Clinical laboratory reports in molecular pathology. *Arch Pathol Lab Med* 131:852-863.
6. *Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guideline*. CLSI, 2009.
7. McGovern MM, Benach MO, Wallenstein S, Desnick RJ and Keenlyside R (1999). Quality testing in molecular genetic testing laboratories. *Journal of the American Medical Association* 281:835–840.
8. *Molecular Diagnostic Methods for Infectious Diseases; Approved Guidelines – Second Edition*. CLSI, 2008.
9. *Molecular Methods for Bacterial Strain Typing; Approved Guideline*. CLSI, 2008
10. *Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline*. CLSI, 2008
11. Nygren E, Wyatt JC, Wright P (1998). Helping clinicians find data and avoid delays. *Lancet* 352:1462-6.
12. Powsner SM, Costa J, Homer RJ (2000). Clinicians are from Mars and pathologists are from Venus. *Arch Pathol Lab Med* 124:1040-6.
13. *Proficiency Testing (External Quality Assessment) for Molecular Methods; Approved Guideline*. CLSI, 2008.
14. *Quantitative Molecular Methods for Infectious Diseases; Approved Guidelines*. NCCLS, 2008
15. RCPA Quality Assurance Programs Pty Limited, *Serology QAP*, viewed 13 October 2010, Australia, <<http://www.rcpaqap.com.au/serology/>>

16. *Real-time PCR in Clinical Microbiology: Verification, Validation and Contamination Control*. Clinical Microbiology Newsletter, 2007
17. Shapiro DS (1999). Quality control in nucleic acid amplification methods: use of elementary probability theory. *Journal of Clinical Microbiology* 37:848–851.
18. *Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline*. CLSI, 2008
19. Valenstein PN (2008). Formatting pathology reports. *Arch Pathol Lab Med* 132:84-94.
20. Wright P, Jansen C, Wyatt JC (1998). How to limit clinical errors in interpretation of data. *Lancet* 352:1539-43.

Further information

Other NPAAC documents are available from:

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