

Submission Guidelines for Nucleic Acid Amplification Tests for Infectious Agents

Section II. Assay Description

For infectious disease applications, packages should be organized into the following sections, labeled, and each section and subsection must be completed. Also the applicable requested information in Section III must be provided.

- A. Methods
- B. Requisition and Reporting
- C. References
- D. Validation Protocol and Data
- E. Assay Verification
- F. Quality Assurance

A. Methods

The Standard Operating Procedure (SOP) should be written such that any technologist can (after training) could refer to the manual, perform the assay, and interpret the results for accurate reporting. An acceptable format should be similar to that found in the Clinical Laboratory Standards Institute (CLSI) (formerly NCCLS) guidelines GP02-A5. At a minimum, please include the following information:

Overview

1. Describe the scientific basis of the test and give an explanation of the assay.
2. Describe the target gene and the region being amplified or analyzed. If available, an oligonucleotide list with sequences, and location of primers and/or probes for the gene of interest should be included.
3. Indicate the target population of the assay, and the indications for testing.

Test procedure

1. Provide the laboratory's SOP (s) for carrying out the assay, including a detailed step-by-step protocol for the entire assay. Include copies of ancillary SOPs pertinent to the steps in the assay.
2. Describe specimen collection, processing, and storage requirements, and specimen rejection criteria.
3. Describe and provide sources for essential reagents and equipment, including reagent recipes (stock and working solutions), final concentrations, and reagent quality control (QC).
4. Describe all calibration and QC materials, including types of controls; sources; how positive controls were verified; preparation and concentration of controls used; storage requirements; number of controls used and frequency of use, in the testing protocol; acceptable control limits; and action(s) to be taken when controls exceed the defined tolerance limit.
5. For all nucleic acid amplification tests, please provide a description of standard workflow, and the procedures that are in place in your laboratory to prevent amplicon contamination.

6. Indicate any technical limitations of the assay, potential sources of error, and troubleshooting protocols.

Controls required for molecular infectious diseases tests

Extraction / Lysis

For optimal QC, at least one specimen per run should contain whole organism (bacterial cells, viruses, parasites, fungi) or, if these are absolutely unavailable, nucleic acid. This positive extraction or lysis control should be included at a low but easily detectable concentration, and should be run through the entire extraction and test process. Additional amplification controls or target controls, such as those that consist of nucleic acid but are not extracted (i.e., introduced into the run after the extraction step), are optional. A negative extraction control should also be included in each run. This control should ideally consist of a known negative specimen in the same matrix that is being tested, and it should go through the entire extraction and amplification process as a test sample. This control will monitor potential contamination that could occur during the extraction and amplification processes.

Inhibition

An appropriate inhibition control must be incorporated into each assay and specimen, to ensure that patient samples are free of amplification inhibitors that could lead to false negative results. Alternatively, data must be provided that demonstrate the absence (or only rare occurrence) of inhibition, in each specimen type. If the extraction method is well established, with supportive evidence in the peer-reviewed literature, these references can be submitted together with the laboratory's own validation data. For each specimen type, the data set should include at least several hundred drawn from the literature, plus 100 samples directly tested in the submitting laboratory that demonstrate that the method is performing accordingly to expectations. The specimens tested in the submitting laboratory should contain low but easily detectable amounts of target that are extracted and assayed, with results provided to that show no loss in detection. If independent supportive evidence is not available, the laboratory needs to provide more extensive in-house data. Typically, 500 samples are adequate to verify that inhibition is occurring in less than 1% of the samples. It should be noted that data obtained assayed from the same matrix containing different but similar analytes are acceptable, if they are needed to attain the required number of samples. NOTE: it is acceptable for the inhibition control to also serve as the extraction control.

Examples of inhibition controls include, but are not limited to:

1. specimens spiked with an exogenous nucleic acid control that is detected in a separate assay;
2. specimens spiked with a control nucleic acid containing primer-binding sites identical to the target, but with a heterologous probe-binding internal sequence;
3. specimens dispensed into at least two aliquots, with one of the aliquots spiked with a low level of the positive control and tested in parallel.

In all cases, an expected value and range should be developed for the inhibition control, and results should be interpreted accordingly.

Contamination

The use of a no-template control product or negative control is a primary mechanism by which to demonstrate the absence of general reagent/assay material contamination with amplified target. Water or buffer can also be used as a no-template control in a reaction tube that contains all of the reagents for amplification, but does not contain nucleic acid template. Water or buffer can also serve as a control for reagent contamination. The use of carriers such as tRNA, glycogen, or DNA can be used to detect low level nucleic acid contamination.

Proper molecular workflow is essential in minimizing the risk of DNA/amplicon contamination. The SOP should describe the strategy used for separating pre-and post- amplification areas, as well as the implementation of methods to minimize amplicon contamination (such as the use of unidirectional workflow and aerosol-resistant pipette tips).

The inclusion of including additional negative controls is strongly recommended whenever the prevalence of the target agent is routinely expected to be high, e.g., in HCV screening of intravenous drug users, or patients with suspected hepatitis.

Results and interpretation

1. Describe expected results, including those for positive, negative, indeterminate, and inconclusive samples.
2. Give examples of all calculations needed to produce interpretable results and, if applicable, the algorithm used for interpretation.

B. Requisition and Reporting

Include a sample requisition form and provide reports (in the laboratory's official report format) for all applicable findings: positive (quantitative or qualitative), negative, indeterminate, and inconclusive. Also, provide sample reports that include all applicable technical limitations, interpretative text, and any disclaimers required by the Federal government for tests utilizing Analyte-Specific Reagents (ASRs). Molecular amplification assays for microbial detection that are not probe-based, such as SYBR Green real-time PCR with melting curve analysis, require the use of a secondary method in order to report a result as confirmatory. Non-probe based assays can be used as screening assays but must be confirmed with an alternative method, i.e., a probe-based, hybridization-based, or sequence-based method. In the absence of such confirmation, positive results are considered presumptive, and this should be clearly indicated on the report. A sample report with appropriate wording should be submitted.

C. References

Provide copies of pertinent literature references that describe the scientific basis and clinical utility of the assay. In addition, please submit up-to-date test kit package inserts, if the test is commercially distributed, and/or package inserts for any commercially prepared reagents.

D. Validation Protocol and Data

The validation data must demonstrate that the performance of the assay is sufficiently robust for clinical diagnosis of human samples. Before addressing each of the subtopics below, please summarize why this new or modified assay is needed, the general approach that you have taken to validate the assay, and how the study was carried out. Be sure to include a detailed summary of the final validation results and conclusions drawn, and documented approval by the responsible Director/Assistant Director of the laboratory. For real-time amplification assays, please submit representative data for Ct or similar threshold values, nucleic acid sequenced-based amplification (NASBA) graphs, or similar data, and examples of routine assay runs that include applicable calibrators and QC. Please note that validations using authentic clinical specimens are preferred, but spiked clinical samples are acceptable. For validation studies using spiked clinical samples, spiking material should be whole organisms. If these are absolutely unavailable, spiking with target nucleic acid is acceptable. However, spiked diluents or other non-specimen matrices are not acceptable substitutes.

1. Provide data demonstrating the analytical specificity of the assay. The assay should not detect related organisms, organisms that can produce similar symptoms, or other organisms that can be present in the specimen matrices(s) to which the assay will be applied. If any cross-reacting organisms are noted, they should be clearly specified in the application, as well as on the patient report of test findings. Please provide data showing that no cross-reactivity with these types of organisms occurs when the target organism is assayed at clinically relevant concentrations. Conversely, the assay should be able to detect all strains or subtypes of the organism that contain the target analyte. If the assay has been designed to detect a species or subtype, data should be submitted from at least two strains for verification purposes. If multiple sub-types or genotypes exist for the organism being assayed, include examples of each type or for a representative range of subtypes.

2. Provide data demonstrating the limit of detection (LOD, or analytical sensitivity) of the assay, based on using a number of samples sufficient to establish a statistically valid result. What is the lowest quantity of target that can be reproducibly detected? A dilution series of a known concentration of the target (preferably whole organism) should be spiked into negative clinical specimens, to establish the LOD. For virology applications, the LOD in genomic copy number is required. The LOD is the lowest amount of analyte in a sample that can be detected but not necessarily as an exact value. In molecular methods and quantitative molecular methods, the LOD is the lowest concentration of analyte that can be consistently detected (typically, in at least 95% of

samples tested under routine clinical laboratory conditions). The lowest amount of target must be assayed at least three times to ensure that it can reliably be detected. This concentration must yield an assay value that can be reproducibly distinguished from values obtained from samples that do not contain the analyte. For quantitative viral assays, the limit of quantitation (LOQ) should be determined. The LOQ is the lowest amount of analyte in a sample that can be quantitatively determined with acceptable precision and accuracy, under the stated experimental conditions. Quantitation based on plaque titrations of viruses is not acceptable. When an assay is validated in multiple sample types (matrices), a separate dilution series should be performed for each matrix, although some sample types may be grouped. If it is unclear whether you need to independently spike different matrices (for example, different types of body fluids) please contact CLEP for advice.

3. Provide data showing inter- and intra-assay reproducibility.

If you assay the same set of samples several times, in a single run, how closely do the results agree? If you assay the same set of samples on different days, how closely do the results agree? When you perform reproducibility studies, you must run at least three authentic clinical or spiked clinical samples in triplicate, in the same batch, to demonstrate intra-assay reproducibility. These same samples can be run on three different days to demonstrate inter-assay reproducibility. Alternatively, a single positive control run repeatedly over many days (15 or more) will also fulfill the latter requirement. Please note that, if spiked, the three samples should differ in target concentration (at least one log difference between one sample and the next). In addition, if different instruments and/or technologists will be used to perform the assay, you should demonstrate the assay's consistency across these variables, by utilizing the different instruments and/or using different technologists in the inter-assay comparison runs.

E. Assay Verification

You must provide independent verification that the results obtained by your laboratory are correct. This can be accomplished in several ways. In order to appropriately verify the assay, the lab needs to carry out a randomized, blinded validation study, by one or more of the following methods:

1. Comparison of the results obtained to those of a "gold standard" assay that has been in generally accepted use and/or is FDA-approved;
2. Comparison of the results obtained to the results from another CLEP-approved assay, either in your lab or in another lab;
3. Comparison of the results obtained from an assay of spiked clinical specimens to the predicted results based on the spiking values.

Please submit results from *at least* 30 positive samples for each specimen type, along with 10 negative samples and all controls used in the assay. Ideally the 40 samples should be authentic clinical specimens, but spiked samples are acceptable. If spiked samples are used, at least 10 samples should be at or close to the LOD (maximum, 10-fold above that level). If authentic clinical samples are used, at least 10 of the 30 positives should be weak positives, if the methods used allow this determination. For each assay, document the number of specimens tested, the source and the type of specimen assayed. If multiple sub-types or genotypes exist for the organism being

assayed, please include an example for each type, or for a representative range of subtypes. When non-probe based assays are used, please submit data obtained from a secondary confirmatory method (see Section II, Requisition and Reporting). For quantitative assays, please provide data across the full range likely to be encountered in clinical samples. Please submit representative examples for the study (including some high-quality original printouts of actual test runs), a condensed summary of the raw data (such as Ct values), and a complete description of how all results were interpreted. When appropriate, 2x2 tables, showing the qualitative results compared to results obtained by another method are encouraged. Any discrepant results need to be explained. If your laboratory has difficulty in obtaining sufficient samples of a particular specimen type, or any other problems in fulfilling the validation requirements, please contact CLEP for guidance.

F. Quality Assurance

Identify the critical steps in the test procedure and the QC measures taken to control and monitor assay performance for consistent and reliable results. In cases when no New York State proficiency test is available for an assay, describe the mechanism that will be put in place to verify the accuracy and reliability of test results at least twice a year, as required by the March 1, 2008 Standards Of Practice, Quality Assessment Sustaining Standard of Practice 3 (QA S3): Ongoing Verification of Examination Accuracy. The SOP or quality assurance plan should include the number of samples included in the blinded panel, the passing rate for the test, and corrective actions that would be taken in the event of inadequate verification of the assay and individual analyst performance. If your laboratory is testing multiple organisms using the same extraction and PCR methods for the same specimen type, it is acceptable to verify competency of staff using one representative organism in an internal proficiency testing panel.

Section III. Additional Information for Specific Molecular Applications/Assays

1. Change of Intended use for FDA-approved tests.

For FDA-approved tests that are already being conducted in your lab, but for which your lab now seeks an off-label modification, we require a reduced validation study.

Establishment of analytic sensitivity in the clinical matrix is required. Validation data must be submitted from a dilution series of organisms in matrix, to demonstrate whether the LOD has changed from the non-modified assay. Ideally, a side-by-side comparison is performed, between the modified assay and the non-modified assay. Data should be provided from a blinded study with *at least* 20 positive samples (with 10 near the limit of detection) and 10 negative samples for each analyte. As above, these samples can be spiked specimens if true clinical samples are not available. However, spiked laboratory diluents or buffers are not acceptable. Data for specificity and reproducibility are not required.

2. Use of sequence databases for comparison and identification of bacteria, mycobacteria, and fungi.

Broad range sequencing Standard Operating Protocol (SOP) requirements for bacteria, mycobacteria, and fungi.

Broad-range sequencing is defined as the use of conserved sequences within phylogenetically informative genetic targets (i.e. 16S rDNA, 28S rDNA, internal transcribed spacer regions, or gyrase B) to identify bacteria, mycobacteria, or fungi. For additional information on specific viral sequencing, subtyping, or genotyping applications, please contact CLEP. Laboratories that apply for approval to carry out identification and subtyping using DNA sequencing and comparisons to sequence databases will be required to provide the following information in the SOP (using Section A Methods (above) as a guide).

The following information must be submitted for review:

- A complete description of controls that are used in the assay. A negative amplification control should be included in each assay. Positive amplification controls are not necessary as all reactions should be positive. In addition, sequencing controls to assess the quality of the sequencing reaction should include both a negative and positive in every run. The sequencing negative control may be water or buffer and the sequencing positive control could be a purified plasmid that is supplied with some commercially available sequencing kits.
- A description of the criteria used to determine the minimum sequence length that is required to properly carry out identification. For short read lengths, at least 300 bp or full-length target sequence if shorter than 300 bp must be utilized for comparison and must span a region of variability. CLSI guideline MM18-A can be consulted for further information on sequence data parameters that should be considered in all sequence-based identification assays.
- A description of the process used to analyze sequence data, including review of electropherograms, the expected read length of the sequence, and the protocol used for interpreting ambiguous base calls.
- A detailed algorithm describing the range of identity matches that will be reported (i.e., genus, species, strain, serotype, or genomovar level, and the per cent identity at which the assay is deemed to fail in identification).
- If database information is proprietary, and other information is not available, an explicit comment should be added in the client education materials, indicating that the database(s) have not been reviewed or approved by the New York State Department of Health.
- Sample reports must be submitted, detailing the wording of the reporting algorithm described in the SOP.

The use of a single public database is not acceptable for bacterial sequence-based identification assays. If searches are performed using the BLAST algorithm, identification parameters should be set so that only query results consisting of sequence coverage of 100% along the entire length of the sequence are used for identification. If use is made of a commercial database that has been verified by sequence-based methods, this single source can be sufficient. If an in-house developed database is utilized, the sequence must be verified by comparison to results obtained by conventional microbiological methods, and/or by a CLEP-approved assay or multiple public databases.

Validation requirements of isolates for broad range sequencing of bacteria, mycobacteria, and fungi.

Section II D, Validation Protocol and Data; section E, Assay Verification; and section F, Quality Assurance (above) should be consulted as guides for the steps used to monitor performance of the assay which should be outlined in the validation material. Below are additional guidelines that need to be followed for proper validation of these assays:

- If an application includes a sequenced-based assay tested directly from a patient specimen rather than a culture/isolate, sensitivity and specificity data should be submitted. Data demonstrating specificity and the limit of detection for use on bacterial, fungal, or mycobacterial isolates are not required.
- Reproducibility studies should include testing of 3 different authentic isolates tested in singlicate on 3 different days. If possible, each of the 3 authentic isolates should be tested by a different laboratorian.
- Data should be submitted from at least 30 representative organisms tested in a blinded fashion, and should include the phenotypic identification of each organism. If 30 representative organisms cannot be obtained, call CLEP for guidance. Data can be submitted in the following format:

Sample #	Commercial Database Match (Organism)	Input/Seq. Length (bp)	Output/match Length (bp)	#Mis-match (bp)	% Match/ID	Sequence Identification	Culture/ Gold Standard Identification	Final Identification
1496	<i>Ralstonia pickettii</i>	436	436	0	100%	<i>Ralstonia pickettii</i>	<i>Ralstonia pickettii</i>	<i>Ralstonia pickettii</i>
	<i>Ralstonia solanacearum</i>	436	436	19	95.6%			
1644	<i>Haemophilus parahaemolyticus</i>	460	460	7	98.5%	<i>Haemophilus parahaemolyticus</i>	<i>Haemophilus parahaemolyticus</i>	<i>Haemophilus parahaemolyticus</i>
	<i>Haemophilus paraphrohaemolyticus</i>	460	460	10	97.8%			
1555	<i>Streptococcus bovis</i>	468	468	0	100%	These four species cannot be differentiated by 16S rDNA sequence analysis	<i>Streptococcus bovis</i> group	<i>Streptococcus bovis</i> group
	<i>Streptococcus lutetiensis</i>	468	468	0	100%			
	<i>Streptococcus infantarius</i>	468	468	0	100%			
	<i>Streptococcus equinus</i>	468	468	1	99.8%			
	<i>Streptococcus alactolyticus</i>	468	468	27	94.2%			

- After satisfactory review of the SOP and validation data, the application will be tentatively approved. The laboratory will then be required to participate in a specialized assessment administered by the Wadsworth Center, consisting of a blinded panel of 10 data sets or organisms. Full approval will be granted after successful completion of the assessment. If performance on the specialized assessment is unsatisfactory, CLEP will contact the laboratory to provide guidance, and to discuss additional training necessary in order for the laboratory to obtain approval to perform broad-range sequencing.

3. Subtyping

Submissions for approval to perform subtyping using DNA sequencing and comparisons to sequence databases (which need to be identified and defined), should provide data on as many different species or sub-types as possible in the validation panel, including at least three isolates for each species or sub-type represented in the database. However, if database information is not available, an explicit comment should be added in the client education materials, indicating that the database(s) has/have not been reviewed or approved by the New York State Department of Health. For additional information on validation requirements for specific viral sequencing, subtyping, or genotyping assays, please contact CLEP.

4. Multiplex analytes (especially in multiple sample types).

We appreciate that multiplex assays can present a difficult situation for design of a validation study, since the assaying of 40 samples (30 positive and 10 negative) for each analyte in each specimen type can be a large and expensive endeavor. Samples can be spiked with multiple organisms, so as to reduce the number of samples required. We suggest contacting CLEP for guidance prior to submission of a package with multiplex analysis having four or more simultaneous targets.

5. Prognostic Viral Genotyping Assays

Viral genotyping assays do not absolutely require inhibition controls and specificity studies. For assay verification, the blinded panel should consist of at least 30 positive samples for each specimen type and this panel needs to contain sufficient representations of genotypes that will be reported by the laboratory.

Rare Genotypes

We appreciate that certain genotypes that are rare may be difficult to obtain, however, validation on these genotypes must be performed if they are to be reported. A deficiency in rare genotype specimens in the assay verification study may be overcome by including rare genotype specimens in inter- and intra-assay reproducibility studies to increase confidence that they can be genotyped accurately. If rare genotypes are not available, reports will require a statement indicating the genotypes that have been properly validated and that validation is ongoing for the rare genotypes. Additionally, the laboratory will need to provide a plan for ongoing validation of these rare genotypes and a written plan needs to be included in the SOPM for handling rare genotype specimens until fully validated.

Subtypes

For genotypes with multiple subtypes, assay verification of these subtypes is not required if these subtypes are not clinically different from the primary genotype.

Mixed infections

The methods need to include a description of what steps will be taken in the event of a mixed infection and how these results will be reported. If mixed infections will be reported, proper assay verification needs to be performed. Assay verification studies may be performed by preparing mock specimens containing mixes of genotypes of various concentrations. For example, two pure samples can be used to prepare mixes of the genotypes at various ratios (e.g., 1:1, 2:1, 1:2, 4:1, 1:4).

Section IV. Other Considerations

Resubmissions (after initial review).

Once the package has been reviewed, the laboratory will receive either an approval letter or a letter requesting additional information. In order to facilitate our response to resubmissions, we request that these be as clear and well organized as possible.

Please provide a point-by-point response to the reviewers' comments, as well as the appropriate supporting documentation. If revisions to the procedure manuals were requested, please submit entire new versions, and clearly indicate where the changes were made.