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MINIREVIEW

In-House Nucleic Acid Amplification Assays in Research: How Much Quality Control Is Needed before One Can Rely upon the Results?

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Over the last 20 years, nucleic acid amplification tests (NAATs) have become a major tool for detection of microorganisms, for diagnostic testing, and for research purposes in the field of infectious diseases. NAATs offer significant sensitivity and speed compared to culture and do not require viable organisms. However, validated, commercially available, U.S. Food and Drug Administration-cleared assays exist for the following microorganisms: *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, methicillin-resistant *Staphylococcus aureus*, group B streptococcus, *Legionella pneumophila*, human immunodeficiency virus, hepatitis B virus, and hepatitis C virus. Some of these tests are for very limited indications, for example, the methicillin-resistant *Staphylococcus aureus* assay is intended only for use with nasopharyngeal swabs as an infection control tool.

There are also a number of so-called analyte-specific reagents commercially available for clinically relevant pathogens and pathogenicity factors like herpes simplex virus, Epstein-Barr virus, cytomegalovirus, *Streptococcus pyogenes*, and *Bordetella pertussis* and the genes for *vanA*/*vanB* and *mecA*, respectively. Next to these relatively closed and standardized kit concepts, the use of NAATs for research purposes has expanded dramatically. These assays range from those that are well validated to not validated at all, yet these assays are frequently used and cited in the literature. A review of the current literature on the association of a particular microorganism and a particular disease frequently reveals inconsistent results, even apparently when the same methods are used.

Although NAATs offer the promise of exquisite sensitivity, theoretically allowing for detection of a single organism in a clinical sample, both false-negative and -positive results can and do occur. There can be problems with sensitivity, specificity, and contamination, which can be secondary to a very large number of technical issues, as listed in Tables 1 to 6. Some of the more common problems in context with NAAT-based studies of microorganisms and disease associations are described below.

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FALSE-POSITIVE RESULTS DUE TO CONTAMINATION

The possible association between human herpesvirus 8 (HHV-8) and a direct role in Kaposi's sarcoma pathogenesis is very important. The presence of this potentially oncogenic virus in semen donated for use in artificial insemination would be critically important to know. However, the prevalences of HHV-8 DNA in prostate and semen as measured by NAATs range from 0 to over 90% (32). A recent study (32) assessed interlaboratory sensitivity and reproducibility in the analysis of blinded, identical experimental panels, each consisting of 48 samples composed of semen specimens from artificial insemination donors, human immunodeficiency virus-infected patients, and positive and negative controls. As no commercial, validated assay is available, each laboratory followed its standard procedures for HHV-8 PCR; thus, procedures varied from laboratory to laboratory. Results of this study pointed out a twofold problem. First, of 10 experiments done in five laboratories, 5 experiments from three laboratories had evidence of NAAT contamination; all instances of contamination were in the context of nested PCR procedures. Second, in the experiments with no false-positive results, HHV-8 DNA was detected in 3 (8%) of the 37 semen specimens but in only 3 (1.6%) of the 184 NAAT analyses performed on these specimens. This suggests that HHV-8 DNA is present in semen at concentrations that may be too low to allow its consistent detection.

Another example is the association of *Chlamydia pneumoniae* and atherosclerosis; the organism has been detected by various NAATs in 0 to 100% of atherosclerotic lesions (5, 15). This variation was also shown to be true when identical samples were analyzed by means of different NAATs or the same NAAT. Two recent multicenter trials compared various DNA extraction methods and PCR protocols for the detection of *C. pneumoniae* DNA from endarterectomy specimens. In the first study (3), a panel of identical clinical atheroma specimens and controls were sent to 9 laboratories in Europe and the United States; the reported positivity rates for detection of *C. pneumoniae* DNA by various NAATs ranged from 0% to 60%. There was poor concordance between the different laboratories, as only 25% agreed on one specimen and there was no correlation between the detection rates and the sensitivity of NAATs used.

In a subsequent multicenter study (2), aliquots of carotid artery plaques were prepared by means of three extraction

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TABLE 1. Issues to be considered and sources of possible error in connection with preanalytical procedures for NAATs

Step	Issue(s) to consider and indicate	Pitfalls and comments
A	Sampling and transport conditions	Specimen transport time and conditions can have an effect on the overall analytical sensitivity; labile target molecules may easily degrade upon prolonged storage at room temperature
B	Sample storage conditions	For certain microorganisms within biological samples, the efficiency of cell lysis may depend on storage conditions
C	Aliquoting and/or splitting procedures	Splitting is problematic, since the target organism may be unevenly distributed; the splitting process itself is also a common source of exogenous contamination

methods. The nested PCR assay subsequently used was and is one of the most widely used assays. This assay was one of four PCR protocols recommended in a consensus paper published in 2001 by the Centers for Disease Control and Prevention (Atlanta, Ga.) and the Laboratory Centre for Disease Control (Ottawa, Ontario, Canada) in an attempt to propose criteria required for performing in-house NAATs (8). Of 240 PCR results analyzed, only 5 (2%) were positive for *C. pneumoniae* DNA (2). After exchange of DNA extracts between the laboratories, the overall positivity rate was 5% out of 720 analyses, which was actually lower than that seen in the negative controls (8%). Not one of the specimens was positive when all of the atheroma extracts were reamplified by means of a NAAT targeting a different gene followed by hybridization with a *C. pneumoniae*-specific probe. Statistical analyses demonstrated that positive results were most likely explained by amplicon carryover during the nested PCR as well as amplicon introduction during DNA extraction.

These studies clearly demonstrated that even experienced laboratories can have problems with contamination, especially when using nested NAATs. Since the late 1990s, this PCR format has emerged as one of the most frequently used variation of conventional PCR. The nested PCR format has been used in the majority of studies to increase sensitivity and specificity. Amplicons (PCR products of the target gene region produced during the single- or first-step PCR) are reamplified using a second, inner primer pair. The nested PCR format requires the opening of vials potentially containing highly positive fluid (millions of copies of the target gene of interest) to set up the second "nested" PCR, thus providing a high risk of carryover contamination. The results of these multicenter studies applying nested PCR (2, 32) suggest that many of the reported prevalence rates based on this or similar technologies most likely represent background contamination rates rather than true positives.

FALSE-NEGATIVE RESULTS DUE TO LOW SENSITIVITY

An external quality assessment for molecular detection of *Bordetella pertussis* was recently conducted in 11 European laboratories (25). All 11 laboratories used in-house-developed NAATs, as no commercial assay is available. The participants were asked to report whether the samples were positive or negative for *B. pertussis* and whether another *Bordetella* species was detected. The first testing panel (dilutions of three *B. pertussis* clinical isolates and negative controls) revealed no false-positive results, but the reported detection limits varied between 4 log grades (e.g., from 3 to 30,000 CFU/ml), suggesting that false-negative PCR results with clinical specimens may be a major problem.

FALSE-POSITIVE RESULTS DUE TO LOW SPECIFICITY

The same external quality assessment for NAAT detection of *B. pertussis* has sent a second testing panel (dilutions of *B. pertussis*, *Bordetella holmesii*, *Bordetella hinzii*, *Bordetella bronchiseptica*, and negative controls) to 9 laboratories. Only one laboratory performed the tests with 100% specificity for *B. pertussis* by use of the *B. pertussis*-specific target gene pertactin. The eight other laboratories, which used IS*481*-based assays, reported positive results for *B. pertussis* for the samples spiked with *B. holmesii* and *B. bronchiseptica*. These unexpected false-positive reactions for *B. pertussis* by the majority of the laboratories suggested the presence of a homologous sequence in the strains of these two species used (25). The false-positive results for *B. pertussis* for samples containing *B. holmesii* and *B. bronchiseptica* strains in this quality assessment program further suggest that the specificity and positive predictive value of IS*481*-based PCR assays for the diagnosis of pertussis may be compromised and bias results of epidemiological and clinical studies alike.

Four months after publication of this study (25), a paper was published (13) describing the construction and application of an internal amplification control for detection of PCR inhibitors applied to the NAAT diagnosis of whooping cough by amplification of a fragment of *B. pertussis* IS*481*.

C. pneumoniae has also been linked to multiple sclerosis (MS) (35), and as seen with the association of *C. pneumoniae* and atherosclerosis, NAAT results have been inconsistent from study to study. A large multicenter study sent 30 and 22 cerebrospinal fluid (CSF) samples from patients with MS and controls, respectively, to four different laboratories in the United States and Europe. One of the four sites reported detecting *C. pneumoniae* DNA in 73% of CSF samples from patients with MS and in 23% of CSF samples from patients with other neurologic diseases. However, specimens analyzed at the other three sites were all negative (18). Subsequently, the primers used at the site reporting the high number of positives were demonstrated to have high sequence similarity to human DNA, as determined by BLAST search and amplification of human DNA (36), which suggested that they were not uniquely specific for *C. pneumoniae*. In addition, cycling conditions used in this assay were likely to allow for nonspecific product formation and probably detected various human genes which are present in clinical specimens containing human cells.

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TABLE 2. Issues to be considered and sources of possible error in connection with sample preparation and DNA extraction for NAATs

Step	Issue(s) to consider and indicate	Pitfalls and comments
A	Method	State all modifications and the version of a kit protocol; most commercially available kits provide numerous diverging protocols
B	Amt of specimen	Use concn and/or wt; unusual metrics do not allow for evaluating sensitivity and data comparison
C	Elution vol	Missing information does not allow for back calculations and makes interstudy comparison impossible
D	No. and quality of ENCs[a]	"Water only" ENCs without carrier DNA might miss low amplicon carryover; the same is true for too-small no. of ENCs
E	No., quality, and concn of EPCs[b]	Too many and/or too highly concentrated EPCs can be a contamination source, as can certain formulations (e.g., amplicon, irrespective of whether cloned or not)
F	Integrity and amt of DNA	DNA might get lost during repeated thawing-freezing cycles or extraction; amplification of genes other than the target does not prove integrity of an intact target region and rather provides an indication of specimen quality and quantity
G	Quality and concn of ICO[c] if used	To check for a successful DNA extraction and/or inhibition, ICO might be included at the extraction level; primers and probes used to amplify ICO should have attributes similar to those of targets; if the ICO concn is too high, complete or partial inhibition is not ruled out; if the concn is too low, ICO might get lost during extraction and inhibition is falsely assumed
H	Anticontamination strategy	Basic recommendations include separate rooms with laboratory coats, safety cabinet, pipettes plus aerosol-resistant pipette tips, gloves, and racks; wrong airway pressure conditions favor contamination; all reagents used should be subdivided into small aliquots[d]

[a] ENC, extraction-negative control.
[b] EPC, extraction-positive control.
[c] ICO, internal control.
[d] A good and still up-to-date review on NAAT laboratory design and workflow can be found in reference 24.

WANTED: ETIOLOGIC AGENTS

Based on various NAATs, *C. pneumoniae* has also been associated with cyclosporine-induced hypergingivismus (37), chronic skin wounds (20), age-related macular degeneration (17), and chronic anemia (27), to mention only a few. As these conditions all have markedly different pathologies and pathogenic mechanisms, to ascribe them to one microorganism is not biologically plausible. This problem is not restricted to *C. pneumoniae.*

Rickettsia helvetica is another good example of a rather exotic, difficult to diagnose microorganism which was associated with various conditions of markedly different pathologies. First, and based on nested PCR assays, *R. helvetica* was linked to chronic perimyocarditis resulting in sudden cardiac death in young people (28). DNA of *R. helvetica* was detected in the pericardium and in a lymph node from the pulmonary hilum of one patient and in a coronary artery and the heart muscle of a second patient. Nested PCR results were confirmed by means of sequencing. Sequencing would have also confirmed a positive result due to amplicon carryover contamination and rather ensured specificity. Other confirmatory methods used in that study were electron microscopy (EM) and immunohistochemistry (IHC), both of which suffer from objectivity in terms of how to interpret findings like "*Rickettsia*-like structures." IHC gives rise to cross-reactions between antitarget antibodies and nontarget proteins in complex biological samples, producing nonspecific signals. The interpretation of EM pictures is also not convincing, and results must therefore be interpreted with caution.

Some years later, the same group reported having detected *R. helvetica* DNA in various tissue types obtained during the autopsies of 2 patients with sarcoidosis (30). Three nested PCR assays targeting the 17-kDa outer membrane protein gene, the 16S rRNA gene, and the citrate synthase gene (the first two were also used in the study described above) of *R. helvetica* were applied. There was no concordance for *R. helvetica* DNA positive results achieved by the various assays applied to 12 tissue specimens. Confirmation was again done by sequencing PCR products, IHC, and EM.

Based on a microimmunofluorescence technique, others found no evidence of *R. helvetica* infection in Scandinavian sarcoidosis patients (33). In that study, sera from 20 well-characterized sarcoidosis patients were investigated for anti-rickettsial immunoglobulin G antibodies with *R. helvetica*, *Rickettsia conorii*, and *Rickettsia typhi* as antigens. None of the investigated sera displayed detectable titers of anti-rickettsial immunoglobulin G antibodies.

In 2005, and again using the same three nested PCR assays for detection of *Rickettsiae* as in the studies above (28, 30), the authors claimed to have demonstrated not only *Rickettsia* spp. but also *C. pneumoniae* and *Bartonella* spp. in sclerotic heart valves of patients undergoing aortic valve replacement by various nested PCR assays (29). The authors report detection of DNA of *Rickettsia* spp. and *C. pneumoniae* in 17 (20.2%) and 22 (26.2%), respectively, of 84 pathological aortic valves. In six cases (7.1%), these two organisms coexisted. In 3 of the 15 control valves, *C. pneumoniae* DNA was also detected. The authors suggest that *Rickettsia* spp. also have a role in the pathogenesis of aortic valve disease. In that study, results were confirmed by observation of not only "*Rickettsia*-like" but also "*Chlamydia*-like" structures by EM and IHC.

Other examples of inconsistent NAAT-based associations are atherosclerosis linked to *Helicobacter pylori* (22), herpes simplex virus, cytomegalovirus and Epstein-Barr virus (14, 21), and periodontal microorganisms (6, 10, 11). A number of "syndromes of yet unknown etiology," including chronic fatigue

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TABLE 3. List of issues to be considered and sources of possible error in context of assay design for NAAT

Step	Issue(s) to consider and indicate	Pitfalls and comments
A	Target gene(s)	Note whether there is conservation within a species; no. of copies within the bacterial genome can influence assay sensitivity (34); the same is true for multiplex assays (amplifying multiple targets simultaneously)
B	Primers and probes: database and date of each analysis, accession no., alignments, software used	Elementary searches and results of sequence alignments may vary by date; sequences chosen years ago might be inappropriate; note whether sequences were taken from standard databases (which may contain some wrong, extra, or "missing" nucleotides) or whether sequencing has been performed on local isolates and/or type strains
C	Published primers and probes	State yr of design of original assay and proceed as under step B; otherwise state that this information is not available for any reason
D	Published NAATs	State any modifications done to a published NAAT

syndrome, fibromyalgia, and sarcoidosis, have been also linked to *Mycoplasma* (9, 26), Borna disease virus (10, 31), HHV-8 (4, 7, 12), and *Propionibacterium* spp. (38). All of these studies have claimed detection of these organisms using various NAATs.

WE KNOW THE PROBLEM: WHAT CAN WE DO ABOUT IT?

It is a hallmark of research that findings might not necessarily turn out identical and always conclusive between different groups. However, findings and conclusions based on in-house NAATs have frequently created contradictory and conflicting data, as described above. If findings of one research group remain unique despite the effort of others to reproduce these results, the alert reader should not accept these results at face value and take a closer look at the methods.

When one carefully reads many of these publications, often little to no information is provided in terms of how the NAAT used has been validated. As shown in the tables, there are a number of critical steps in terms of identifying sources of error in context with specificity, sensitivity, and false-positive and -negative findings; application in a particular specimen type, population, or clinical disease; and intra- and interlaboratory reproducibility of NAATs. Steps where problems can arise include: (i) preanalytical procedures, sample preparation, and DNA extraction (Tables 1 and 2); (ii) assay design, format, and set-up in terms of sequences, concentrations, and conditions chosen (Tables 3 and 4); and (iii) interpretation, confirmation of results, and quality control issues (Tables 5 and 6).

Especially, insufficient prerequisites to avoid contamination, application of techniques highly prone to amplicon carryover, such as the nested PCR format, or even other techniques requiring handling of amplicons appear to be a major problem (2, 19, 20). The potential problems with contamination were recognized shortly after the introduction of PCR as a diagnostic technique in 1986 (23). Nested PCR techniques were developed in the late 1990s and were used in the majority of studies to increase sensitivity and specificity: amplicons are reamplified using a second, inner primer (pair), running two PCRs subsequently. Vials potentially containing millions of specific amplicons have to be opened to set up the second reaction as well as for product detection, thus providing a high risk of carryover contamination.

Thus, even if one assumes that a particular NAAT was 100% specific and sensitive in terms of sequences and/or chemistry chosen, the results might still be biased by undetected false positives due to amplicon carryover. Wearing gloves in separate, dedicated areas for the various steps during the amplification process as well as the usage of dedicated pipettes and stuffed tips, e.g., does not guarantee reliable results, even though the positive and negative controls react as expected (2).

The use of a NAAT already published in the literature, even if recommended (8), does not rule out these concerns. The list of potential pitfalls is long, and failure to address a single point, as listed in Tables 1 to 6, correctly might lead to loss of sensitivity but also to serious specificity problems, resulting in flawed results followed by misleading conclusions and biased associations.

Diagnostic testing for microorganisms based on molecular microbiological techniques has become more and more complicated; unfortunately, the complexity of the technology may be beyond the expertise of many in the field. To determine whether a particular NAAT used in a particular study is appropriate or not requires a high level of background knowledge in genetics, including the steadily growing library of microbial genomic databases. And the field is changing and expanding rapidly. Thus, a NAAT established 10 years ago and designed at that time with the best of information and knowledge available may not necessarily be state of the art today. In addition, over the years, PCR technologies have also steadily improved. Real-time-based platforms currently seem to offer numerous advantages over conventional NAATs. However, every month, papers continue to be published using methods for which there is evidence that they are inadequate at various levels by current standards.

DO WE NEED A SPECIAL REVIEWER FOR GENETIC ISSUES?

Journal editors need to give their best attention to the peer review process with special focus on the molecular methods. As an example of the complexity of this issue, how many reviewers check all submitted sequences by doing the align-

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TABLE 4. List of issues to be considered and sources of possible error in context with assay setup for NAAT[a]

Issue	PCR type(s)	Consider and indicate	Pitfalls and comments
Format	Conventional	Single, semi-nested, or nested PCR	Employing a unidirectional workflow, negative air pressure conditions, and/or dedicated safety cabinets are essential prerequisites for nested PCR reactions; dUTP/AmpErase (see below) cannot be used in nested assays, since the template for the second amplification would be destroyed
	Real-time	Platform: instrument and software version	Current state-of-the-art, closed, automated system, preferentially including amplicon inactivation; combines amplification, product detection, and quantification in a single step
		Chemistry: TaqMan probes, LightCycler (FRET) hybridization probes, Biprobes, molecular beacons, Scorpions, etc., SYBR green with/without probe, melting curve analysis	Various chemistries for different purposes with variable specificity are available, e.g., application of SYBR green without probe applied on clinical samples might be unspecific despite melting curve analysis, since SYBR green stain has a high affinity for any amplified double-stranded DNA; although yet not officially stipulated, “diagnostic” NAATs have to rely on sequence-specific analysis methods; detection by gel electrophoresis or SYBR green is thus unacceptable
Concn and conditions	Conventional and real-time	Quality and effective concn of oligonucleotide components	Quality of oligonucleotides can be assessed by MALDI-TOF analysis or analytical HPLC and concn can be determined spectrophotometrically (OD_{260}); in practice, there are significant lot-to-lot or supplier-to-supplier variations; quality of oligonucleotides can also suffer from inadequate storage and handling; resulting problems could be complex Some well-balanced multiplex NAATs could heavily suffer from residual salts in the supplied primer or probe preparations Typical melting point of the real-time PCR hybridization probe may change
		dUTP/AmpEraseUNG	Amplicon generated by master mixes containing dUTP instead of dTTP is destroyed by the enzyme UNG during the first denaturation cycle in each newly set up PCR, allowing only genomic DNA (containing natural dTTP)—and not carried over amplicon—to be amplified
		Cycling conditions (times, temps, no. of cycles) for denaturation, annealing, and extension	To achieve a higher stringency concerning binding of primers, some conventional assays are based on the “touchdown” technique—a high annealing temperature during the first cycles to increase specificity is followed by decreasing annealing temperatures, e.g., every second cycle, for efficient amplicon amplification; if primer sequences are suboptimal or start temp too low, unspecific product may be generated
		State any changes made to a published assay	Inappropriate concn might lead to unspecific product formation or “destroying” the analytical sensitivity of well-balanced multiplex applications (e.g., Mg^{2+} concn)
Controls at PCR level and standards for quantification	Conventional and real-time	No., quality, and concn of TPC	Too many and/or too highly concentrated TPCs can be a contamination source, as can certain formulations
		No. and quality of NTP	NTPs contain master mix only and are used to exclude contamination at the PCR level; if too few NTPs are included, contamination might be missed
		Inhibition control: internal controls, genomic target DNA, target spiked into specimens’ DNA or amplification of human gene sequences, etc.	If amplification of a gene different from the target (or some other oligonucleotide spiked in) is used, the primers and/or probe should have attributes similar to those used for the target; otherwise, inhibition cannot be ruled out; has it been demonstrated that the presence of inhibition control does not negatively influence the efficiency or analytical sensitivity of the NAAT for the respective “native” target gene?
		Standards	Did each quantitative NAAT include a standard curve (prepared on how many replicates of how many dilutions?), or was the standard curve imported from a separate run? In the absence of codified standards, absolute quantification data may be highly variable!

[a] FRET, fluorescent resonance energy transfer; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; HPLC, high-pressure liquid chromatography; OD_{260}, optical density at 260 nm; UNG, uracil *N*-glycosylase; TPC, template-positive control; NTP, no-template control.

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TABLE 5. List of issues to be considered and sources of possible error in context with interpretation and confirmation of results of NAAT

Step	Issue(s) to consider and indicate	Pitfalls and comments
A	Interpretation: what was considered a positive/negative analysis and what was considered a positive/negative result	How many replicates of those tested had to be positive for the sample to be considered positive? How was the assay-specific cutoff value defined, e.g., based on clinical data or presentation of the patients; microscopic, culture, or serological results; or just on the assay's lower detection limit?
B	Confirmation: (i) by DNA reextraction and NAAT repetition; (ii) by amplification of different gene; (iii) by independent method	Neither hybridization (irrespective of whether a probe, amplicon, or primers are used) nor dot blotting or sequencing represents an adequate methodology to confirm a specimen as truly positive or negative for a target gene, since these methods would also confirm a positive result accomplished due to amplicon carryover contamination; the specified methods rather ensure specificity of the amplicon produced during PCR and, partly, might enhance assay sensitivity

ments themselves? Although several journals, including those published by the American Society for Microbiology (1) and the Infectious Diseases Society of America (16), require that these data be provided by the researcher, this is often not the case. Even if these data are provided, it is not guaranteed that they will be correct. New platforms and technologies, which may be appropriate for one application but inadequate for another, are frequently used in the wrong context. As an example, researchers might use a real-time PCR-based NAAT but, for detection of the amplified product, only use SYBR green chemistry followed by melting curve analysis without a probe. When applied on complex biological specimens, this can pose a serious specificity problem. Besides technical details, such as concentrations and conditions, how many reviewers pay attention to what was done to ensure adequate anticontamination strategies?

Following the example of using dedicated reviewers to ensure proper statistical methods, as is done by several journals,

TABLE 6. List of issues to be considered and sources of possible error in context of assay validation for NAAT

Issue	Issue(s) to consider and indicate	Pitfalls and comments
Sensitivity	Analytical sensitivity (detection limit on purified target DNA) and sensitivity in mock-infected specimens (detection limit if target is spiked into particular specimen or infected specimen)	Analytical sensitivity as well as sensitivity on spiked specimens might not be representative for NAAT applied on clinical samples for numerous, partly unknown reasons (e.g., intra- or extracellular location of the target organism in clinical samples, relatively high amount of human genomic DNA in a typical clinical specimen); the detection limit in a given background (human) DNA level is critical; NAATs validated for one specimen type may not perform equally on a different specimen type
	Sensitivity in clinical study—comparison to "gold standard"	Many NAATs never have been validated and compared to the "gold standard" (e.g., culture) in clinical settings
Specificity[a]	Wild-type strains	Note whether any wild-type strains or other species were tested; dependent on how conserved the target region is (or how comprehensive the database used for its choice) within the species, the NAAT might or might not pick it up in a clinical sample; note whether the evaluation strain panel contained local isolates of the target organisms or a battery of type strains (e.g., ATCC)
	Specificity compared to "gold standard"	Note whether any attempts were made to also analyze specimens by the method considered the "gold standard"
Positive and negative predictive values	Data on the performance of the assay in a given population	Understanding sensitivity and specificity is not the be-all and end-all because they do not address the problem of the prevalence of disease in a particular population; e.g., the positive predictive value of a 99.9% sensitive and specific NAAT changes drastically from 50% of individuals testing positive in a low-incidence population to 99% of people testing positive being truly positive in a high-prevalence population; the negative predictive value also changes depending on the prevalence of the disease
Reproducibility	Intralaboratory	Note whether replicates were tested and how they correlated (day-to-day or week-to-week variations)
	Interlaboratory	Note whether there are any data providing information in terms of the application of the NAAT in other institutions and whether multicenter evaluation data are available and what the quality/level of validation of that data is

[a] See assay design and setup issues (Tables 3 and 4).

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we propose that there be an additional independent reviewer for genetic purposes. To this end, authors would need to submit all the necessary supplemental material on their molecular methods along with their manuscripts. A scoring system based on a checklist for the various issues listed in the tables could ensure that all NAAT methods in submitted manuscripts are evaluated the same way.

CONCLUDING REMARK

Unless appropriate proper methodological sections in research papers are guaranteed, any data generated based on questionable NAAT protocols will themselves be questionable.

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