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Review

Control Strategies in Molecular Diagnostics: A Tiered, Risk-Based Framework for Accuracy, Reliability, and Real-World Use

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Abstract

Controls are fundamental to ensuring accuracy and reliability in molecular diagnostics, yet their roles are often oversimplified or conflated with broader quality assurance frameworks. As molecular testing expands from centralized laboratories to point-of-care (POC) and over the counter (OTC) settings, the design, implementation, and interpretation of controls must evolve to address diverse operational environments and clinical risks. This review introduces a comprehensive framework for understanding control strategies in molecular diagnostics, integrating internal, external, and orthogonal controls within a tiered, risk-based testing model. We categorize diagnostic systems into three tiers—screening (OTC/POC), confirmatory laboratory testing, and reference-level or adjudication testing—and examine how control requirements scale with analytical complexity, user variability, and clinical impact. Across these tiers, controls serve distinct but complementary roles, including verification of assay functionality, mitigation of contamination, maintenance of cross-platform consistency, and resolution of diagnostic uncertainty. We further analyze common failure modes in molecular diagnostics, including sample-related errors, inhibition, contamination, and interpretation challenges, and map how specific control strategies mitigate these risks. Regulatory perspectives from the U.S. Food and Drug Administration (FDA), Clinical Laboratory Improvement Amendments (CLIA), International Organization for Standardization (ISO), and World Health Organization (WHO) guidelines are discussed, highlighting the shift toward risk-based and context-dependent control design rather than rigid, one-size-fits-all requirements. Importantly, we address the balance between control burden and clinical utility, emphasizing that excessive control implementation may increase system complexity without proportionate gains in diagnostic value particularly in decentralized settings. Emerging trends, including artificial intelligence (AI)-assisted diagnostics and decentralized molecular platforms, are also explored as transformative approaches to enhancing control integration and result validation. We propose that a tier-adaptive, risk-based control framework is essential for next-generation molecular diagnostics, enabling accurate, scalable, and user-centered testing systems. This perspective supports the development of robust diagnostic platforms that maintain analytical integrity while improving accessibility and real-world performance.

Keywords: controls; internal control; external control; molecular diagnostics

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2. Introduction

The world of diagnostic devices is evolving rapidly, and so is the need for rapid, more accurate, and easily attainable diagnostic tests. Molecular diagnostics encompass a wide range of tests used to detect infectious diseases, cancers, genetic disorders, and various DNA, RNA, protein, and biomarker targets [1]. Molecular and other categories of diagnostics rely on *sensitivity*, the likelihood of a positive

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test result with a diseased patient, and *specificity*, the likelihood of negative test result with a healthy patient [2]. These tests are typically described as dichotomous, producing two outcomes: positive or negative [1,2]. Dichotomous tests are simple, informative, and accurate, being a favored testing method and are often used for quantitative testing by implementing a numerical cutoff [2]. Nonetheless, many forms of molecular diagnostic tests use multiple forms of control such as internal and external controls in addition to negative and positive controls. Thus, despite these analytical safeguards, the final clinical output remains dichotomous.

Defining terms such as negative and positive control, internal control, external control, reference standard, and gold standard are important to clearly understand the context of this review. Many molecular diagnostic reviews focus on reference standards and gold standards within the field and how they are evolving, but [internal] negative and positive controls themselves are never discussed in detail. These controls are commonly used in all types of molecular diagnostics and are almost an unsaid requirement, which could contribute to why they are rarely reviewed.

Negative controls are used to rule out contamination and to validate if the sample being applied to the test does not have the disease being tested for [3]. The positive control serves the opposite function, confirming that the assay can detect the target and generate a positive result when present [3,4]. Similarly, internal controls are run at the same time as negative and positive controls and have various functions such as chemistry validation and overall device function validation [5,6]. External controls are typically run independently from the initial testing, often in a separate setting to confirm initial results and that the test is functioning properly [7,8]. A reference standard is a similar molecular diagnostic test of high sensitivity, specificity, and accuracy utilized to verify test results received from a different testing method [9]. And lastly, the gold standard is often the molecular diagnostic's optimal and chosen method for consistently receiving diagnostic truth results, for example bacteria culture in diagnosis of Group A Streptococcus [9]. Reference and gold standards are often similar, with gold standards evolving over time and sometimes utilized as a reference standard.

As molecular diagnostics and OTC and POC tests evolve, the use of controls and their applications are becoming even more important, especially as NAATs become more sensitive and specific. This raises the question of whether both internal negative and positive controls are necessary and if comparator testing, such as reference and gold standards, are needed as future tests become more sensitive and accurate than the standards.

3. Defining Tiered Testing

There are tires of molecular diagnostic testing that are categorized based on the level of risk, who and where the test is being used, and its purpose. The following section looks at each tier individually. A summary of the tiered framework and associated control strategies is presented in Table 1.

Table 1. Tiered framework for molecular diagnostic testing and associated control strategies.

Category	Tier 1: Screening (OTC/POC)	Tier 2: Confirmatory Laboratory (CLIA Lab)	Tier 3: Reference / Adjudication (Research/Reference Lab)
Purpose	Rapid identification; triage for further evaluation	Confirm diagnosis and guide treatment decisions	Resolve uncertainty; support high-stakes decisions and research
Intended use and setting	Community, home, retail; minimal infrastructure	Clinical laboratories; controlled environment	Reference labs; highly controlled environments

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Typical users	Non-professional / lay users	Trained laboratory professionals	Highly trained specialists
Examples of tests	Rapid tests, NAAT, isothermal amplification	PCR, qPCR, multiplex NAATs	NGS, digital PCR, orthogonal methods
Control strategy focus	Ensure test runs correctly and is interpretable	Ensure accuracy, precision, reproducibility	Ensure analytical truth and consistency
Control types	Internal/procedural controls; built-in controls; digital interpretation	Internal controls; positive/negative controls; extraction controls	Orthogonal controls; reference materials; computational controls
Key characteristics	Fast, simple, user-friendly; higher variability risk	High analytical performance; regulated	Highest rigor; advanced analysis
Clinical impact	Low to moderate (screening)	High (guides decisions)	Very high (definitive/adjudicative)

3.1. Tier 1 Testing

The first tier of diagnostic testing is screening tests which are used in large-scale populations and include rapid tests, OTC tests, and at-home tests. These tests allow for individuals who are at risk for a disease, need reoccurring tests for chronic diseases, or are asymptomatic, to be screened earlier without a clinical visit and have better health outcomes [3]. Protection of individuals beyond the tested population is another purpose of screening to prevent transmission of infectious diseases [4]. Two outcomes are possible for screening tests, negative and positive [4]. Positive test results for screenings are indicative that the individual needs confirmatory testing and treatment by a licensed physician [3,4]. Negative screening results tell that the individual is not likely to have the disease or biomarker and does not need further medical attention [4]. False-negatives and false-positives are commonly produced by screening tests; thus, tests with high sensitivity and high specificity that have a coinciding control are less likely to have false test results and have elevated accuracy that can be comparable to confirmatory tests [4]. In this tier, control strategies are primarily integrated into the design of the assay to support usability in non-laboratory settings. Procedural or internal controls, such as sample adequacy indicators or control lines, are incorporated to confirm that the assay workflow has been completed and that the system is operational. Built-in chemical or biochemical controls are also included to monitor overall assay functionality during use. In addition, many point of care and OTC devices incorporate digital or algorithmic components within reader-based systems to standardized signal interpretation [10]. These controls may be presented as user visible indicators or embedded within the system, depending on the design and intended use of the test [3].

3.2. Tier 2 Testing

The second tier of diagnostic testing includes confirmatory, validation, or verification testing which often refers to all diagnostic tests that are performed in a clinical laboratory setting. The results obtained from molecular assays in this tier of testing are significantly more accurate, reliable, and reproducible, making it ideal for clinical laboratory settings and for giving valid information for treatment in patient care decisions [11]. In tier 2 testing, well characterized reference standards and orthogonal methods are essential to establishing testing with confirmatory safeguards. The actionable results received in this tier of testing should be proportionate to its intended use and clinical risk, where rigorous confirmation rather than exploration is used to make prognosis, guiding treatment decisions that could involve irreversible interventions [11]. Two types of molecular diagnostic assays are categorized in the United States for clinical laboratory testing, laboratory developed tests (LDT) and in-vitro diagnostic (IVD) medical devices [12]. A laboratory using FDA cleared/approved IVD medical device test must verify that it is analytically specific and sensitive, precise, accurate, robust

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and has a reportable range [11]. IVD test kits that have been modified by a clinical diagnostic laboratory are regulated as in-house tests or LDTs, requiring further validation to document new performance of the test [12]. Second tier molecular diagnostics include isothermal amplification, polymerase chain reaction (PCR), next-generation sequencing (NGS), sanger sequencing, nucleic acid hybridization techniques, microarrays, northern and southern blots, and mass spectrometry [5,12]. Control strategies in this tier are more structured and externally reinforced to support higher analytical rigor. External positive and negative controls are included at the run level as part of quality insurance practices [7,8]. Internal amplification controls are incorporated within assays to accompany each reaction, while extraction controls are used during sample preparation steps to track nucleic acid recovery and processing performance [5,6]. In addition, lot-to-lot verification processes are applied to evaluate consistency between reagent batches and maintain consistency and comparability of assay performance over time [7,11]. These control elements are integrated into laboratory operations and are checked by quality management frameworks.

3.3. Tier 3 Testing

The third tier includes reference, sequencing, and clinical endpoint adjudication (CEA) testing. Clinical endpoint adjudication is a process that has been standardized to evaluate the safety and efficacy of medical devices and pharmaceutical products in clinical trials [13]. The purpose of clinical endpoint adjudication testing is to have consistency, accuracy, independence, analytical neutrality, and a blind evaluation of presumed clinical events [13]. While CEA is used in general, it most frequently occurs in clinical trials related to cardiovascular studies to provide an independent, systematic, and standardized review of reported events [13]. Relevant clinical experts compose the CEA committees and are supported by an endpoint office (EPO) responsible for governing clinical documents [13]. In this tier, control strategies extend across analytical, methodological, and interpretive components of testing, showing the high clinical and regulatory impact of controls. Orthogonal controls are a defining feature of third tier testing and are used to compare results across independent methods or platforms to resolve uncertainty and reduce systematic bias [14]. These approaches are important in situations where no single method can be assumed to represent the true disease state, requiring consistency across methods to support result validity [14,15]. Reference materials and traceability standards play a central role in this tier by allowing comparability of results across laboratories, platforms, and time. Standardized reference materials with known characteristics are used to calibrate assays, verify performance, and ensure alignment with externally defined benchmarks [7,16]. These controls support reproducibility in the lab and are essential for establishing consistency in studies and clinical trials. In next generation sequencing and other data intensive applications, control strategies extend beyond the wet lab environment into bioinformatic and analytical pipelines. Pipeline controls include validation of alignment algorithms, variant calling processes, and data filtering steps to ensure that computational processing does not introduce systematic error or bias [12]. The use of curated datasets, simulated controls, and benchmarking frameworks allows for continuous verification of pipeline performance and detection of algorithmic drift over time [12]. Controls in this tier are not limited to detecting isolated failures but are designed to ensure overall system reliability.

3.4. Function of Controls

In molecular diagnostics, controls are used to ensure that test results are reliable by identifying specific points where errors can occur, while gold standards provide a true reference used to evaluate diagnostic accuracy through measures such as sensitivity and specificity [10]. However, this framework assumes that the reference standard represents the true disease state, which isn't always the case, as imperfect gold standards can introduce bias and lead to misclassification of results [1]. When considered within tiered testing frameworks, the function of controls becomes closely tied to the purpose, risk level, and testing environment of each tier. In first tier testing, which consists of screening, rapid, and over the counter assays used in large populations to identify individuals who

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may need further evaluation [4], tests are dichotomous, producing positive or negative outcomes that prioritize sensitivity for early detection [3]. At this level, the dominant risks are sample-related failures and user error, which lead to false-negative results [10]. These risks are addressed through embedded procedural controls that confirm sample adequacy and verify that the test has functioned correctly, ensuring that negative results are not due to failure of the testing process [2,11]. Additional risks such as reagent degradation or instability are mitigated through built-in chemical or system controls, as well as performance verification strategies that confirm assay components remain functional under expected use conditions [2,11]. In second tier testing, the dominant risks shift toward analytical failures, including inhibition, contamination, reagent degradation, operator variability, and instrument malfunction. Inhibition, caused by substances such as hemoglobin or anticoagulants interfering with amplification reactions, is detected using internal amplification controls that verify that amplification is occurring as expected [6]. Environmental interference and contamination, which are sources of analytical error, are identified through negative controls that detect unintended amplification in the absence of target material [17], while nonspecific amplification and off target product formation can generate false results, contributing to analytical error and the misinterpretation of results [18]. In third tier testing, the dominant risks shift from analytical performance to challenges in interpretation, cross platform consistency, and validation in the absence of a definitive reference standard. Controls are designed to address uncertainty from complex datasets and highly sensitive detection methods. One major challenge is method-dependent variability, where different platforms or analytical approaches may lead to disagreeing results. This is addressed through orthogonal testing strategies, where independent methods are used to confirm findings and reduce the chance of systematic bias [14]

3.5. Internal vs External Controls

To monitor overall test performance, separate samples with known expected results—also called external controls—are tested with, but outside of, patient samples. These controls are used to monitor overall assay performance. For complete-assay positive controls, the control material should challenge the full workflow and should resemble a patient specimen as closely as practical [19].

To verify that an assay is functioning properly for a specific test, internal controls are built into, or run within, an individual test. Internal amplification controls may be designed as either endogenous or exogenous controls, depending on the assay format [20]. When an internal control is introduced before sample preparation, it can monitor extraction as well as downstream amplification and detection steps [19]. In an era where molecular assays are attuned to a higher level of reference standards, traditional internal quality control approaches are no longer sufficient for guaranteeing reliable clinical results [21,22]. Preventing laboratories with LDTs and IVDs from producing internal results that are inconsistent and mislead patient management decisions; calibration stability, biological truth, and alignment with the reference system must expand beyond precise regulation to actively monitor internal controls [21,22]. Thus, rather than using commercially produced material for internal controls, they should mimic patient samples as closely as possible to reflect real clinical conditions as much as possible without forsaking quality and accurate results [21,22].

Internal controls function as safeguards that provide immediate confirmation that each individual test has proceeded correctly. They allow for detection of sample specific failures such as inhibition, insufficient nucleic acid input, or reaction failure [6,11]. In-sample internal controls do not replace external positive and negative controls, but they provide specimen-level evidence that the workflow was adequate for result interpretation [23]. Because they are integrated directly into the assay, they are effective at identifying errors that could lead to false negative or invalid results. However, their embedded nature limits their ability to detect broader trends across multiple runs [24].

External controls are implemented at defined intervals as part of run level or batch level quality control procedures. These controls provide a broader evaluation of assay performance across multiple samples over time. This makes them effective for identifying systematic errors such as

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reagent degradation, instrument instability, and environmental variation that may not be identified by internal controls alone [8,17]. While internal controls enable continuous monitoring within individual tests, external controls allow for the detection of changes and patterns in assay performance but rely on appropriate scheduling, frequency, and adherence to quality control practices to ensure effectiveness [8]. A risk-based quality control plan should also consider the specimen, test system, reagent, environment, and testing personnel, because failures in any of these areas can affect result reliability [25].

Another important consideration is the visibility of controls to different users within the testing system. In point of care and decentralized environments, internal controls are usually designed to be user-visible, such as control lines or indicators that confirm proper test function, supporting usability and reducing operator dependent error. In some decentralized molecular systems, built-in procedural controls include both an internal process control and an electronic control, while external controls may be recommended with each new shipment and for new or untrained operators [26]. In contrast, external controls and quality checks operate within laboratory workflows or instrument software and may not be directly observable by the end user. Instead, they serve as part of a broader quality management framework that ensures consistency and reliability across testing systems [16,27]. Effective quality control in molecular diagnostics does not rely on a single type of control, but rather on the integration of internal and external systems that address different sources of error across both individual tests and overall assay performance.

4.0. Failure Modes in Molecular Diagnostics and Where Controls Intervene

Imperfections are inevitable in molecular diagnostics and knowing when, where, and how certain diagnostic tests fail is key to understanding where controls intervene. For molecular tests completed in a clinical laboratory, sample type, sample preparation, collection methods, time of specimen collection, handling conditions, storage temperature and transportation methods are all possible failure modes [28,29]. Depending on the organism and method of testing, timing of sample collection matters as some requires long incubation periods within the human body [28]. Timing of sample collection may also relate to what part of the body the sample is being collected from and what type of test is utilized, examples include collecting samples in the morning, while fasting, before taking certain medications, etc. Molecular assays are particularly sensitive to suboptimal pre-analytic conditions, where 85% of all laboratory errors are caused by poor pre-analytic conditions, with majority of them occurring outside the laboratory [29]. More specifically, DNA rapidly degrades when deoxyribonucleases (DNases) are present [29]. While DNases play an important role in vivo in protecting against pathogens and regulating apoptosis, in vitro they degrade DNA. Best practice is to store native specimens for DNA at −20 °C or lower for longterm- preservation and dried specimens at ambient temperature [29]. Additionally, depending on temperature, Tris–EDTA buffer purified DNA is stable up to years [29]. Specimens containing intact RNA is required for RNA analysis as degradation by ubiquitous Ribonucleases (RNases) occurs quickly and is difficult to remove or inactivate [29]. RNA preservation requires sample storage at −80 °C unless RNase inhibitors or stabilization solutions are used [29]. While these errors can be fixed with following proper procedures, utilization of an internal or external control alongside the sample enables identification and localization of analytical failures.

Another failure mode in all tiers of testing is false-positive and false-negative results. False-positivity usually occurs when contamination is present within a laboratory or the test reagents. Non-specific amplification in nucleic acid amplification tests (NAATs) is another cause of false-positive results, which occur not only when primers bind to non-target DNA or RNA, but occur with poor primer design, unsuitable annealing temperatures, excessive primer concentrations, or high cycle numbers [18]. False-negative results are also another concern in NAATs that are often difficult to recognize as they can occur because of multiple biological and technical factors [28]. One factor influencing false-negative results is genetic variation, preventing primer binding and need DNA sequencing if missed by PCR or other nucleic acid amplification methods. False-negative results can

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also occur when samples are mishandled, poor sample collection methods used, or inhibitory materials added to a clinical sample [28]. As NAATs, typically PCR and other similar methods, often serve as a reference standard or a comparator for many other clinical LDTs, routine use of negative and positive controls are essential mitigating failure modes. For diseases that are rare and infrequently tested, controls are often not available for LDTs and require validation by having the sample tested in the same manner by another certified laboratory [28].

Once failure modes in molecular diagnostics are understood, methods to remedy and mitigate is essential. Failure mode and effect analysis (FMEA) is one effective method utilized within clinical laboratories, focusing on risk-management and discovering the source of failure [30]. Majority of clinical laboratories focus on reactive methods such as writing incident reports, conducting audits, and external quality assessments (EQA) which are productive but not valuable [30]. This method of resolution appears reasonable considering how highly automated, data intensive, and informatic-dependent laboratories are today. However, this also leaves laboratories vulnerable to failure modes that quality control cannot recognize. Reactive methods detect previously occurring errors, while many other errors go unreported or unnoticed as individual mistakes are focused on rather than system flaws, limiting awareness of underlying risks [30]. FMEA improves system design within the laboratory, therefore preventing errors. The FMEA method is successful as it involves mapping each step of a process, identifying possible stepwise failure modes, prioritizing risks for mitigation, evaluating the significance, likelihood, and detectability of each failure [30]. Lastly, the most notable step is implementing controls to reduce or eliminate risks, by providing comparison against an optimal reference in case of assay failure [30].

5. Control Burdens vs Incremental Risk Reduction

Meaningful patient care, rather than maximizing technical rigor, should guide how decisions are made for diagnostic testing, including such decisions to add controls in terms of checking quality or confirmation [2]. The treatment threshold theory (P_{TT}) explains the risk and benefit, financial expense, time, discomfort, complexity, and chance of user error, for treating those with disease and without disease based on obtained test results for which the disease was designed [2]. The most informative tests are those that have intermediate range for pretest probability of diseases, where results influence clinical decisions; when outside of this range, the test results should not determine treatment outcomes [2]. Benefits obtained from adding controls can advance a post-test probability across a treatment or no treatment threshold only if the analytical accuracy results in significant improvement of the test [2]. On the contrary, testing is no longer valuable and introduces operational errors when there are additional controls, therefore increasing overall test burden [2]. As a result, this burden reduces access and adoption of the test by widening inequities and increasing complexity and cost in decentralized and OTC tests [2]. Controls in excess create unwarranted confidence without producing meaningful improvements in treatment outcomes. Proportional, risk-based control strategies define actual quality improvement and balance reductions in misclassifying risk while preserving accessibility, usability, and real-world effectiveness [2]. The relationship between control burden and clinical value is illustrated in Table 2.

Table 2. Relationship between control burden and clinical value in molecular diagnostics Conceptual model illustrating how increasing control complexity yields diminishing returns beyond optimal balance.

Control Burden Level	Description	Impact on Clinical Value
Very Low	Minimal or no controls; high risk of undetected failure modes	Low reliability; high risk of false results
Low	Basic procedural or internal controls; limited verification of assay function	Improved usability but still vulnerable to analytical errors

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Moderate (Optimal)	Balanced use of internal and external controls tailored to assay complexity and risk	Maximized clinical utility; reliable results with acceptable complexity
High	Multiple layered controls, including redundancy across assay steps	Marginal gains in accuracy with increased operational complexity
Very High	Extensive control systems including orthogonal validation and computational controls	High analytical rigor but increased cost, time, and user burden
Excessive	Overly complex control frameworks exceeding practical requirements	Diminishing returns; reduced usability and accessibility

An imperfect reference standard bias emerges when the reference standard has limitations and does not have 100% accuracy [1]. At times, test performance is reported as Positive Percent Agreement (PPA) or Negative Percent Agreement (NPA) when there is not a true gold standard, exhibited as an imperfect comparator [1]. Similarly, depending on accuracy of a test and disease prevalence, some tests are measured based on the probability that test results reflect true disease status, using positive predictive value (PPV) and negative predictive value (NPV) [1].

A novel test may falsely appear to have poor sensitivity or specificity due to true results of an imperfect reference standard, misclassifying novel test results as errors [14]. Choosing a solid comparator or reference standard is important to prevent distortion and misclassification of test performance that is similar to that of tests with contaminants or limited detection of traditional methods [14].

A tool used to evaluate how well a diagnostic test or clinical marker can distinguish between individuals with and without a condition when a reliable reference standard is available is called the receiver operating characteristic (ROC) curve; where the overall performance of a test is summarized by the area under the ROC curve (AUC) [31,32]. On the y-axis of the ROC curve, sensitivity is plotted against the rate of false positivity (1-specificity) on the x-axis across all cut-off points for what constitutes a normal or abnormal test result [32]. The area under the ROC curve (AUC) with a value of 0.5 indicates no discriminatory ability, while a value pf 1.0 reflects perfect discrimination [31,32]. When examining differences in AUC for diagnostic tests, ROC analysis explains that larger areas under the curve indicate better overall discriminative performance [31,32]. While the ROC curve measures the statistical accuracy of a test, it is not used on a daily basis for those utilizing a test in any setting and it is better used for IVDs undergoing clinical trials or being implemented for the first time as LDT.

6. Human Factors and Real-World Use

In real-world diagnostic settings, human factors significantly influence test performance, especially in decentralized and point of care environments where users may lack formal laboratory training. As diagnostic technologies are increasingly designed for use by individuals without formal laboratory training, variability in user behavior becomes a significant source of error. Although these systems are engineered for simplicity and low risk of error, their performance still depends on correct execution of procedural steps and accurate interpretation of results [33,34]. Human related errors have been widely recognized as a major contributor to diagnostic inaccuracies across all phases of testing, including pre-analytical and analytical processes [17,35]. Cognitive stress contributes to these challenges. Tests that require multiple steps, strict timing, or interpretation of visual outputs increase the likelihood of user error in non-controlled environments [36]. Studies of point of care diagnostics have demonstrated that errors frequently arise from user interaction with the device, including improper sample collection, incorrect procedural execution, and misinterpretation of results, rather than failures of the assay itself [36]. This highlights that analytical performance alone doesn't guarantee accurate real-world results.

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The design and implementation of control systems must account for these human limitations. In decentralized settings, control mechanisms are often embedded within the assay to reduce reliance on user judgment and provide immediate feedback on test validity [10,33]. However, not all control mechanisms translate effectively outside laboratory environments. External controls and structured quality assurance procedures are typically designed for trained people operating within regulated laboratory systems, where adherence to protocols and documentation is expected [7,16]. When applied in non-laboratory settings, these assumptions may not hold, increasing the risk of improper use or inconsistent implementation of quality control measures [8,35,37].

Challenges in real-world diagnostic use arise from the need to balance analytical performance requirements with usability constraints, as increasing system complexity or performance sensitivity can place greater demands on user interaction and device operation [3,36]. This highlights a design tradeoff between human factors and analytical optimization. While laboratory-based diagnostics prioritize sensitivity, specificity, and reproducibility, real-world performance depends equally on usability, clear instructions, and robustness to user variability. Effective diagnostic systems must therefore be designed not only for analytical accuracy but also for reliable performance under the conditions they are used, ensuring that control strategies remain effective across diverse users and environments.

7. Regulatory Perspectives and Gaps

7.1. FDA and CLIA Requirements

In-vitro diagnostic (IVD) devices are defined in section 201(h)(1) of the Federal Food, Drug, and Cosmetic Act (the act) and as well as section 351 of the Public Health Service Act for biological products [38]. Legally marketed IVD devices are classified in the Code of Federal Regulations (CFR) 21 under parts 862, 864, and 866, defining how existing laboratory tests are regulated by the FDA [38]. Based on increasing potential risk to patients, the FDA classifies medical devices into Classes I, II, and III [33,38,39]. FDA clearance through the 510(k) premarket notification pathway requires proof of substantial equivalence to a legally marketed predicate device for Class I and II, and some Class III devices [33,39]. Thus, the Clinical Laboratory Amendments (CLIA) labels diagnostic tests of high-moderate complexity or CLIA waived, with waived tests that are simple and are low risk for erroneous results by unskilled users [33]

From the perspective of the FDA, it is vital to have a well-defined characterization of intended use, as treatment decisions are closely related to clinical risk [38,40]. Clinical validity and utility must be demonstrated along with analytical performance as alone it is not enough [38,40].

As outlined by the FDA's Class II Special Controls Guideline for nucleic-acid-based tests detecting *Mycobacterium tuberculosis* (MTB) complex for respiratory specimens, significant false result risks are associated with tuberculosis tests [34]. The FDA's guideline for MTB requires that control strategies be all-encompassing to decrease the false test result risk via inclusion of negative, positive, and internal controls that have been clearly defined [34]. Negative controls are required to mitigate risks such as non-specific amplification and contamination of reagents, while positive controls are required to monitor reagent reliability and consistent detection of MTB targets [34].

To validate the test as a whole, internal control is required to monitor critical analytical steps ensuring no erroneous test results [34]. The IVD guidelines for this disease are applicable to those who may be creating an IVD for a disease of similar complexity and prone to false test results. Diseases that pose a higher risk to patients and require a higher level of care, such as tuberculosis, need more advanced and reliable testing methods—hence the need for multiple and internal controls to monitor test efficiency.

Current FDA-cleared point-of-care nucleic acid amplification test (NAAT) platforms prove that despite chemistry and workflow differences, each device has embedded internal controls, sharing a common philosophy for regulatory and quality-control [33]. The Abbott ID NOW platform relies on isothermal amplification with internal system and amplification checks that only report negative

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results when internal system and amplification controls pass [33]. To prevent undetected false-negatives if controls fail, the device automatically gives an invalid result [33]. A more comprehensive approach is adopted by Roche Cobas Liat that utilizes real-time PCR, incorporating a positive internal process control that is added at the beginning of the run, separate from the sample, via cartridge and is carried through lysis, extraction, amplification, and detection [33]. This method is used to maintain sample integrity, reagent performance, and amplification efficiency while simultaneously verifying the patient specimen [33]. Cepheid GeneXpert has two different tests; Xpert and Xpress, which use multiple layered internal controls within each cartridge. Controls for sample processing and reagent or probe integrity is also incorporated [33]. This allows for detection of inhibition, extraction failure, or reagent degradation before test results are complete [33]. Designed for highly multiplex syndromic testing, BioFire FilmArray utilizes a nested multiplex PCR approach and sample processing controls that go through lysis, extraction, and amplification [33]. This type of control is critical for multi-target assays given the increased analytical risk [33]. A simplified and more compact molecular IVD is the Mesa Biotech Accula, which includes one single internal amplification control to valid the reaction and preserve ease of use in decentralized settings [33]. These FDA and CLIA waived platforms focus on automated control, clear results, reliable pathogen detection, easily operated, and failure mode detection that is dependable [33]. These devices also show that internal controls are key features to the device and are not supplemental, enabling advanced molecular diagnostic devices to be used in various settings with reliable analytical and regulatory acceptance [33]. It also proves that the FDA requires that the type and number of internal controls used only needs to be relevant to the complexity of the testing device and what is needed to ensure the test is reliable and consistently accurate.

7.2. ISO Requirements

For commercial and laboratory nucleic acid amplification-based in-vitro diagnostic (IVD) devices that are International Organization of Standardization (ISO) 17822:2020 certified require certain laboratory quality standards must be met for detection of microbial pathogens [13]. Guidelines for risk management, process control, and context-appropriate validation are outlined by ISO 17822:2020 as required standards, specific requirements for controls are not stated [13]. ISO 17822:2020 recognizes that reference standards are dependent on the type of test and that there are no one-size fits all whether an internal or external control [13]. Rather, it is better that controls utilize well-characterized reference materials to validate the tests such as positive and negative controls, internal amplification controls, microbial strains or synthetic targets, or processed controls that are applicable to the test [13].

Additionally, International Organization of Standardization (ISO) 17822:2020 mentions that performance evaluation of a test is context-dependent in relation to its intended use, clinical setting, and specimen type or target [16]. Similarly, medical devices that are ISO 13485:2016 certified do not have definitive requirements for controls and reference standards as their performance is evaluated based on a risk-based quality management system [7]. For in-vitro medical devices (IVD), ISO 13485:2016 emphasizes that scientific justification, traceability, and process control are prioritized over utilization of controls and reference standards [7]. The expectation is that manufacturers of IVD’s assess clinical risk, define intended use, and select a proper control and comparator for validation, and lastly document any uncertainties found during design control, verification and validation of the device [7].

Enabling medical laboratories to operate effective quality management systems (QMS) requires a comprehensive framework, outlined by international quality standards, particularly ISO 9001 and ISO 15189 [27,29,41]. These standards recognize that methodological suitability does not guarantee perfect performance or valid results in routine use, emphasizing importance of validation and verification procedures [27,29]. When a test demonstrates fitness for intended use, validation is established, whereas confirmation of characterized test performance before patient testing establishes verification [29]. ISO 9001’s QMS is standardized and applies across different industries, centering

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reference standards or controls as a mechanism to install consistency, decrease risk, and initiate constant improvement [27,29]. The design of controls as explained by ISO 9001 is to prevent nonconforming outputs, showing that tests function as intended [27,29]. ISO 15189 is very similar in its principles but has tailored them for medical laboratories specifically. ISO 15189 requires that laboratory tests utilize internal quality controls, external quality assessment, or use of reference materials [29,41]. Other laboratory requirements include instrument calibration, defined acceptance criteria, personnel competency assessment, and systematic review of results across pre-analytical, analytical, and post-analytical phases [29,41]. In short, based on ISO 9001 regulations, controls are seen as safeguards to regulate test reliability and ISO 15189 values controls as essential clinical tools that validate and verify accuracy of test and laboratory systems [27,29,41].

7.3. WHO Requirements

In the WHO Technical Guidance Series TGS6, in-vitro diagnostic (IVD) medical devices undergoing WHO prequalification, focuses on diagnostic assessment panels, controls, and reference materials as central elements of quality assurance (QA) and quality control (QC) [37]. A risk-based method has been adopted by TGS-6, recognizing both embedded test controls and external panel-based controls [37]. Instead of requiring a single format, TGS-6 allows manufacturers to rationalize what type of controls are used based on the intended use of the device, assay complexity and public-health risk [37]. TGS-6 strongly emphasizes that traceable control materials are well-characterized, commutable, and directly resemble patient samples, and when viable, be linked to recognized reference standards [37].

8. Are Positive and Negative Controls Necessary in Molecular Diagnostic Testing?

8.1. Molecular Diagnostics without Controls, Reference or Gold Standards

Can the accuracy of a test be assessed when there is no control, reference, or gold standard? From a statistical standpoint, yes. Rather than relying on external controls or gold standards, repeating the test from the same individual's sample can separate test performance from hidden disease conditions [42]. With this method, repeated measurements can meaningfully decrease uncertainty in test validity, provided that an individual's disease status remains unchanged across measurements despite test results varying stochastically [42]. Common challenges notated in diagnostic-accuracy studies involve the lack of definitive gold standards and intermediate test results [9]. The issue is that established methods force intermediate test results to be categorized as positive, negative, or simply excluded [9]. Instead of reclassifying or dismissing intermediate results, statistical modeling frameworks such as latent-class modeling can be utilized [9]. The assumption in this model is that underlying diseases determine test results and are directly reflected in intermediate results, therefore reducing bias and better representing real-world diagnostic settings where equivocal findings frequently occur [9]. This model also permits three diagnostic outcomes—positive, negative, and intermediate—improving accuracy and interpretability particularly when intermediate results occur frequently or have clinical relevance [9].

Nucleic acid amplification tests (NAATs) are often more analytically sensitive and specific than reference standards used to validate them (culture, microscopy, or serology), which causes unique challenges for diagnostic evaluation [43]. NAAT performance is naturally underestimated when an imperfect reference standard is used leading to NAAT results being labeled as false-positive [43]. Because of superior test performance of NAATs, latent-class models, repeated-measurement designs, composite reference standards, and agreement-based approaches are better suited than typical approaches [43]. Discordance does not necessarily indicate error in the absence of a control or reference standard; true biological detection by NAATs is reflected, exceeding the capabilities of older methods [43]. Evaluating NAATs without any controls encourages moving away from "truth-based" validation and towards model-based and assumption-explicit reasoning [43]. As reference or

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gold standards are methods used to validate test results, in the IVD world with devices being sold not only to labs but POC and OTC, a test without a control, reference or gold standard (based on testing environment) would not be viable. Even with high sensitivity and specificity, the test would still be subject to false test results without being able to validate where the error is coming from. This would put clinics and individuals at an increased risk of receiving incorrect information about whether treatment is needed for the disease being tested.

8.2. A Tier Adaptive Control Framework is More Valid Than a One-Size Fits All Model

Each tier in molecular diagnostics requires a different control framework relative to the level of testing being conducted and the level of risk associated with the type of test. As explained with Abbott ID NOW, Roche Cobas Liat, Cepheid GeneXpert, BioFire FilmArray, and the Mesa Biotech Accula, negative, positive, and internal controls vary greatly based on the device and the level of risk associated with each [33]. Each device also has a different level of sample processing that occurs within a cartridge, thus requiring different types of controls [33]. More specifically, the types and number of controls used will rely on whether the test is multiplex or single-plex [44,45]. Multiplex tests will require more controls per target being tested whereas a single-plex test would only need one set of controls for a single target [44,45]. When the controls meet tiers of testing and complexity of tests at their own level, accuracy improves and false results are reduced.

9. Emerging Paradigms in Molecular Diagnostics

After the COVID-19 pandemic, the need for more accessible molecular diagnostic tests in all tiers became very evident, inspiring the biotech industry to start thinking outside the box [46]. Molecular diagnostics typically require large, heavy, expensive systems that professionally trained laboratory personnel perform tests on [46]. Nevertheless, an increase in smaller, smarter, more accurate and accessible tests are now becoming available to everyone [46]. Decentralizing molecular platforms will not just increase equity in lower income areas but help reduce diagnostic delays everywhere and greatly reduce the response to future infectious disease outbreaks [46].

Artificial intelligence (AI) is becoming an essential and revolutionary tool all over the world in every field of work. Incorporating AI with molecular diagnostics to aide in data interpretation and automatically validate results could be the key to eliminating false test results and solidifying diagnostic accuracy [46–48]. AI is also excelling in automated pattern recognition and predictive diagnostics, fundamental to improving the area of genomics and multi-omics—transcriptomics, proteomics, and metabolomics [47–49]. More specifically, Next-Generation Sequencing (NGS) produces large genomic sequencing datasets that would be more accurately and quickly analyzed by AI and machine learning (ML) [49]. Through ML, millions of sequencing reads can be aligned, filtered, and annotated while prioritizing genetic variants based on different pathogenic and epidemic relevance [49]. Transcriptomic analysis is another diagnostic tool that AI and ML can be implemented to interpret and manage data in complex RNA sequencing, which is subject to alternative splicing expression variability [49]. Disease classification and treatment response prediction is supported by AI and ML modeling that links disease through identifying patterns in gene expression, co-expression networks, and non-coding RNA signatures [49].

Another area enhanced by AI is qPCR and dPCR analysis, which works to reduce interpretation bias and signal variability [49]. More accurate nucleic acid quantification and improvement between true signals and artifacts from melt curve data is another benefit to ML models that are trained on amplification data [49]. This accuracy obtained from ML and AI models is invaluable to monitoring viral loads, minimal residual disease detection, and infectious disease testing [49].

This advancement in diagnostic technology and data analysis is paving the way to better understanding infectious diseases in order to more accurately diagnose them and improve treatment decisions.

10. Conclusion

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Controls in every tier of testing are essential to molecular diagnostics to validate test results so that those making treatment decisions are accurately informed. The number and type of controls rely heavily on complexity of a test, the testing environment, and risk associated with false results. However, with each added control, burden and risk for mistakes increase for personnel utilizing the test; thus, controls should be added thoughtfully in accordance with what the IVD needs to ensure accuracy and meet FDA, ISO, and WHO regulations. It may seem simpler to not implement any control or reference standard if the developed test has high sensitivity and specificity, though false test results are inevitable and a risk to those managing patient treatments. With an adaptive tier approach to controls, complexity is decreased and accuracy is significantly increased, validating sensitivity and specificity of test results. As artificial intelligence (AI) and machine learning (ML) models become more advanced, molecular diagnostics will benefit greatly with tests that are more accurate in every sense of the word and improve patient outcomes as IVDs become more advanced and readily accessible to individuals in every region.

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