



Widespread omission of aptamer-target binding verification in aptasensor development: Consequences for sensor performance and the need for a K_d gate

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ABSTRACT

Aptamer-based biosensors (aptasensors) are widely explored for analytical and diagnostic applications because aptamers combine molecular recognition with scalable chemical synthesis. Yet aptasensors have achieved limited translational impact, motivating scrutiny of development practices. Critical reviews emphasize verifying target binding of the aptamer construct by determining its equilibrium dissociation constant (K_d , a standard measure of binding strength) prior to aptasensor development to avoid interface-contingent artifacts. We refer to this pre-development requirement as the K_d gate: an auditable, construct-matched determination of K_d in homogeneous, non-denaturing solution under sensing-relevant conditions. Here, we assess compliance with the K_d gate by analyzing 209 aptasensor publications spanning six targets. Only 20 of 209 papers reported any solution-phase verification assay, and only one passed the K_d gate; among the remaining numeric K_d reports, the reported values were weakly constrained or format-contingent and therefore not auditable. As a result, reported sensor-performance claims often cannot be interpreted, transferred, or optimized on a fully rational basis. Although practical feasibility varies across constructs and targets, the observed pattern indicates that technical difficulty alone does not explain the persistent omission. We conclude that voluntary recommendations have not,

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in practice, functioned as an effective gate and that passing the K_d gate should become a mandatory reporting requirement, supported by an auditable checklist for editors and reviewers.

1. Introduction

Aptamers are short, single-stranded oligonucleotides that bind specific molecular targets through non-covalent interactions (Ellington and Szostak, 1990; Iliuk et al., 2011; Keefe et al., 2010; Tuerk and Gold, 1990; Zhou and Rossi, 2017). Selected from sequence-diverse oligonucleotide libraries, aptamers can recognize proteins, peptides, small molecules, and cell-surface markers, sometimes with binding strength and specificity comparable to those of antibodies. Because aptamers are produced by chemical synthesis, they can be readily resynthesized and modified without the batch-to-batch variability inherent to cell-derived biological reagents. These properties have motivated aptamer-based assays in both homogeneous formats, in which all components remain in solution, and heterogeneous formats, in which the aptamer is immobilized or otherwise interface-confined. Aptamer-based biosensors (aptasensors) couple aptamer recognition to electrochemical, optical, mechanical, or other transduction readouts (Cho et al., 2009; Du et al., 2013; Li et al., 2025; Song et al., 2008; Willner and Zayats, 2007). This combination of binding specificity and chemical programmability made aptasensors attractive as potentially low-cost, stable, and readily designable diagnostic technologies.

Three decades after their introduction, however, aptasensors remain scientifically prolific but translationally stagnant. Hundreds of original aptasensor studies are published each year for applications ranging from clinical diagnostics to environmental and food testing (Nguyen et al., 2026; Stangherlin et al., 2025a). Yet aptasensor-based devices have achieved little regulatory or commercial penetration (Friedel et al., 2023; Liu et al., 2022), relative to the scale of the research literature. To the best of our knowledge, no in vitro diagnostic using an aptamer as the primary affinity reagent has received FDA clearance, and regulatory-authorized aptamer diagnostics remain rare (European Commission, 2026; U.S. Food and Drug Administration, 2026). This contrast between prolific research output and minimal real-world deployment points to a systemic limitation rather than an application-specific bottleneck.

The translational gap has prompted multiple analyses of the practical limitations of aptasensor research (Ardalan and Ignaszak, 2024; Leung et al., 2021; Liu et al., 2014; Mauriz, 2025; Rozenblum et al., 2019; Yoo et al., 2020; Zahra et al., 2022). The discussions primarily pointed to the bottleneck at the device level, emphasizing challenges in sensor construction and deployment, such as interface effects, nonspecific interactions in complex matrices, and difficulties in achieving stable calibration and reproducible performance. In this framing, the dominant path to progress was continued engineering of interfaces, transduction architectures, and assay workflows. Despite three decades of continued methodological refinement along these lines, however, the anticipated translational outcomes have not materialized.

More recent studies suggest that this emphasis on in-development optimization may overlook a more fundamental vulnerability: the aptamer construct used as the recognition element may not reliably bind its intended target. The risk is compounded when the exact construct used in the assay has not been independently verified to support bona fide binding before transduction steps are introduced. This vulnerability also reflects shared responsibility across aptamer and aptasensor development. Aptamer developers must rigorously support the binding claims they report, consistent with already proposed minimum publication standards for de novo aptamer selection (McKeague et al., 2022). At the same time, aptasensor developers cannot assume that those claims automatically transfer to reformatted sensing constructs used under new assay conditions. Recent work also shows that aptamer performance can depend strongly on structural stability under

application-relevant conditions. In complex matrices, components may perturb aptamer folding, promote nonspecific interactions, weaken effective binding, and worsen sensor performance, whereas more structurally stable aptamers can better preserve affinity and analytical performance (Li et al., 2025).

Ease of synthesis, facile introduction of chemical modification, and low production cost may broaden access to aptamers, but such advantages do not make them plug-and-play recognition elements. Several investigations have reported that some apparently established aptamers fail to form detectable complexes in solution, or that apparent binding arises from assay- or interface-induced artifacts rather than genuine molecular recognition, including in an aptasensor-relevant protein system where widely used anti-lysozyme DNA aptamers showed low-micromolar, nonsequence-specific interactions and behaved similarly to randomized or polythymine controls (Álvarez-Martos and Ferapontova, 2017; Bottari et al., 2020; Ding et al., 2022; Hou et al., 2021; Tao et al., 2020; Toma et al., 2024; Zara et al., 2021; Zhao et al., 2022; Zong and Liu, 2019). Such artifacts can include interfacial drift, nonspecific adsorption, and other transduction-related effects that can generate target-dependent signals without establishing a real aptamer-target complex (Garrote et al., 2019). This creates a direct mechanism by which unverified binding can undermine translation.

The situation resembles a familiar engineering problem: when a key component is not independently qualified, system-level performance can appear strong while remaining difficult to reproduce and validate (Hirshorn et al., 2017). In aptasensors, validation often occurs at the level of sensor response rather than at the level of the sensing construct itself. This risk is amplified because aptamer constructs are supplied as sequence-defined chemicals that are generally not functionally certified for a specific target, and because routine construct modifications and assay chemistry changes can alter binding behavior. Accordingly, a sensor response is not, by itself, evidence of the formation of a bona fide aptamer-target complex. Target-dependent signals may arise from adsorption, matrix effects, reporter interactions, surface charging, sensor degradation, drift, and other interface- or assay-contingent effects. When such responses are treated as evidence of recognition without a construct-matched solution-phase determination of the equilibrium dissociation constant (K_d), optimization can converge on signal-amplifying phenomena even when the underlying interaction is weak, absent, or context-dependent. Pre-development solution-phase verification therefore provides the necessary baseline for interpreting device-level behavior and for distinguishing genuine molecular recognition from device-contingent response.

Even when binding is established in solution, determining K_d accurately is nontrivial because K_d values are obtained by nonlinear regression and can be affected by large systematic errors despite high apparent precision. Recent analyses have shown that, without explicit assessment of accuracy, reported K_d values may differ from their true values by orders of magnitude (Wang et al., 2023, 2024, 2025). Accordingly, K_d determinations should be supported not only by measures of precision, such as standard error, but also by accuracy confidence intervals. Where feasible, K_d should also be determined by orthogonal solution-phase methods, and the independently determined values should agree within stated precision and accuracy limits.

Although published demonstrations that widely used ‘aptamers’ are non-binders remain relatively rare, this should not be interpreted as evidence that reported aptamers generally bind their intended targets. Independent quantitative auditing appears uncommon in practice. As shown below, few studies report de novo solution-phase verification before carrying published sequences into aptasensor development. When re-verification reveals discrepancies, these outcomes are often

handled informally or remain unpublished, consistent with broader evidence from chemistry research that formal correction practices do not capture all recognized errors once claims enter the literature (Bordignon, 2025). Large-scale analyses have also documented pervasive sequence inaccuracies and widespread deficiencies in sequence reporting across aptamer publications, further weakening confidence in downstream functional claims (Miller et al., 2022). Accordingly, aptasensor development cannot assume that a published aptamer is a bona fide binder in the absence of independent quantitative verification in homogeneous solution.

Several factors may contribute to this persistent omission. One is perceived lack of necessity: widely reused aptamer lineages may be carried into sensor development on the assumption that previously reported affinity and specificity automatically transfer to reformatted sensing constructs. Another is genuine practical difficulty: no single homogeneous method is universally suitable for every aptamer-target pair, and some small-molecule systems or heavily modified constructs can be challenging to evaluate quantitatively in a fully construct-matched way. These difficulties are compounded by the fact that buffer composition, ionic strength, divalent ions, cosolvents, labeling, and other assay conditions are often changed during sensor development without re-verification, even though such changes can materially affect folding and apparent binding. Thus, technical infeasibility is best viewed as one contributor rather than a complete explanation for the broader pattern observed in the literature.

Against this background, a key question emerges: do aptasensor studies routinely perform independent, quantitative verification of aptamer-target binding in homogeneous solution as a pre-development quality-control step, or is binding commonly inferred from device-level signals and other device- or assay-contingent readouts? In critical reviews and methodological discussions, a recurring theme is that affinity claims intended to support device development should be established quantitatively under homogeneous, surface-free conditions when feasible, and ideally validated by orthogonal methods, because interface-dependent formats can be sensitive to immobilization chemistry, probe density, and related parameters (Bottari et al., 2020; Ding et al., 2022; Hou et al., 2021; Stangherlin et al., 2025b; Tao et al., 2020; Zara et al., 2021). An accurately determined K_d provides a necessary baseline for identifying the binding regime of a proposed sensor and, although the limit of detection (LOD) depends on many factors beyond K_d , remains an important starting point for rational sensor design and evaluation once aptamer specificity has been established. We refer to this pre-development requirement as the **K_d gate: an auditable, construct-matched determination of K_d in homogeneous, non-denaturing solution under application- and format-relevant conditions.**

Because solution-phase verification tests intrinsic recognition independent of downstream transduction, the K_d gate is a general quality-assurance requirement for aptamer use and is especially important for heterogeneous formats, where interface effects can generate target-dependent signals without a transferable aptamer-target complex. Although these expectations have been articulated repeatedly in critical reviews and methodological discussions, they have functioned mainly as guidance rather than as a uniform operational reporting standard. In this audit, we operationalize them through three requirements: homogeneous, surface-free evidence of binding, a construct-matched determination of K_d , and presentation of this evidence in a form that permits independent audit. As a result, the extent to which foundational solution-phase binding verification is implemented in practice remains unclear, motivating both a systematic compliance assessment and the need for a concise reporting framework by which authors, reviewers, and editors can determine whether de novo, quantitative solution-phase verification has been explicitly documented in the publication record.

To determine how pre-development verification is documented in practice, we conducted a systematic assessment of the aptasensor literature. Specifically, we examined 209 peer-reviewed publications

describing aptasensor development for six well-studied targets (three proteins and three small molecules). We evaluated whether they passed the K_d gate under the criteria defined here, that is, whether they reported a de novo, construct-matched determination of K_d in solution. This analysis focuses on the pre-development stage and does not assess device-level considerations and in-development optimization exhaustively.

Across this 209-paper corpus, solution-phase verification was uncommon and rarely reported in an auditable form. Only 20 of 209 papers reported any solution-phase verification assay, and only one passed the K_d gate under the operational criteria used in this audit. These findings indicate that voluntary recommendations have not, in practice, functioned as an effective gate for aptasensor development. We therefore propose that passing the K_d gate be treated as a mandatory reporting requirement and provide a concise checklist to support its implementation in submission and peer review. In this way, the checklist provides a practical mechanism for authors, reviewers, and editors to document pre-development binding verification explicitly and in auditable form, helping ensure that aptasensor development begins from a verified molecular-recognition event rather than from device-contingent signal alone.

2. Evidence requirements for pre-development validation of aptamer constructs

Aptasensor development should begin by passing the K_d gate, defined as an auditable, construct-matched determination of K_d in homogeneous, non-denaturing solution under sensing-relevant conditions. This requirement follows from the nature of aptamers as reagents and from failure modes repeatedly documented in the literature (Bottari et al., 2020; Ding et al., 2022; Hou et al., 2021; Tao et al., 2020; Zara et al., 2021; Zhao et al., 2022; Zong and Liu, 2019). Unlike antibodies, which are commonly provided as functionally qualified biological reagents, aptamers are supplied as sequence-defined chemicals, and routine manufacturing quality control typically does not certify target recognition. As a result, resynthesis of a claimed aptamer sequence does not provide an auditable bridge to prior binding claims, making independent solution-phase verification a prerequisite for device development.

The K_d gate is particularly important because apparent binding can arise without a bona fide solution-phase aptamer-target complex. Substantial evidence shows that many reported aptamers either do not form such a complex in solution or display apparent affinity driven by assay- or interface-dependent artifacts, including electrostatic adsorption, target aggregation, or recognition of tags and auxiliary epitopes rather than the native target itself (Álvarez-Martos and Ferapontova, 2017; Bottari et al., 2020; Ding et al., 2022; Hou et al., 2021; Tao et al., 2020; Toma et al., 2024; Zara et al., 2021; Zhao et al., 2022; Zong and Liu, 2019). Because aptamers are often selected or triaged in surface-associated formats, apparent enrichment may sometimes reflect surface interactions or partitioning artifacts rather than genuine solution-phase binding (Le et al., 2026). Some failures may originate upstream during selection and early characterization; however, even downstream, surface- or interface-dependent effects can produce convincing device-level signals without a transferable aptamer-target complex. These interfacial effects differ in origin and are often strongly format-dependent and coupled, making them difficult to isolate or neutralize with a standard set of controls. For this reason, solution-phase verification is needed not because every surface assay is poorly executed, but because a homogeneous assay removes the surface itself and thereby removes this entire class of interfacial confounders at the source.

Within this framework, pre-development validation has two evidentiary requirements. First, the aptamer sequence should be supported by a homogeneous-solution determination of K_d , reported with sufficient detail to assess precision and accuracy and, where feasible,

corroborated by orthogonal solution-phase methods, consistent with quantitative validation frameworks for aptamer characterization (Le et al., 2026). Independent affinity-binding studies that focus primarily on rigorous solution-phase characterization can provide valuable pre-development evidence for aptamer choice and design (Samokhvalov et al., 2018, 2025; Sun and Zhao, 2018). In this study, however, such evidence counts toward passage of the K_d gate only when it is explicitly reported within the aptasensor-development paper itself. Second, when an aptamer is advanced into a specific sensing format, binding must be established for the construct intended for sensing and under relevant conditions, unless that same construct has already been validated within the same project under those conditions. Accordingly, performing an assay in solution is not, by itself, sufficient to satisfy the K_d gate if the determination is not interpretable, construct-matched, and relevant to the intended sensing conditions. In practice, the solution-phase recognition reagent must match the sensing construct, including any permanent chemical modifications and assembled recognition state, while excluding immobilizing supports and transducer materials. Verification also requires that both binding partners are well defined, that solution conditions do not promote aggregation or nonspecific association, and that the target form is either application-relevant or explicitly justified as a surrogate.

Here and throughout, the term *construct* refers to the solution-phase aptamer reagent claimed to provide molecular recognition, defined by its full nucleotide sequence and any permanent chemical modifications, such as biotin, fluorophores, quenchers, spacers, or redox tags. For constructs that are inherently multipart or that form the recognition unit through assembly, such as split aptamers (Debiais et al., 2020), duplexed structure switching formats (Saunders et al., 2025), or constrained hairpins (Xu et al., 2014), the construct is the complete nucleic acid assembly state present during target recognition, including strand identities and stoichiometry as used. Immobilizing supports and transducer materials, such as streptavidin-coated surfaces, beads, electrodes, nanoparticles, or hydrogels, are not part of the construct for this gate and cannot substitute for solution-phase evidence. For example, if an aptasensor uses a biotinylated aptamer immobilized on a streptavidin surface, verification must be performed on the same biotinylated aptamer in free solution and, if relevant, in its assembled recognition state, not only in the immobilized format (confirming that the analyte does not bind to the aptamer-devoid support is of course a necessary negative control).

Auditable construct matching also requires clear sequence provenance. In some aptasensor literatures, the reported sequence is traced through secondary sources, inconsistently cited, or not linked clearly to the primary report of the aptamer lineage (Miller et al., 2022). Although such citation problems are not the main focus of the present study, they matter because they weaken construct identity and therefore the interpretability of any claimed affinity value. A reported K_d is not fully informative if the exact construct to which it refers cannot be unambiguously connected to the construct used in the sensing study.

Once construct identity is clear, pre-development verification must establish binding existence and strength in an auditable, transferable manner; accordingly, the evidence must be quantitative rather than qualitative. In this audit, “quantitative” means an auditable determination of K_d for the sensing construct in homogeneous solution under defined, sensing-relevant conditions, based on a dataset that constrains the isotherm within an explicit equilibrium model. By contrast, “qualitative” evidence may be consistent with recognition but does not provide such a K_d , for example because it relies on device calibration or other assay-level standard curves, uses indirect or format-contingent readouts, or lacks the concentration coverage needed to constrain K_d . Thus, an assay may be quantitative for analyte measurement while remaining non-quantitative for binding verification. Because K_d is determined through nonlinear modeling of empirical observables rather than measured directly, high precision alone does not ensure accuracy, and determined K_d values can be systematically biased even when

experimental reproducibility is high (Wang et al., 2023, 2024, 2025). Accordingly, qualitative binding claims and device-response proxies such as signal magnitude or apparent sensitivity are insufficient to establish molecular recognition. Determination of K_d under homogeneous conditions is therefore indispensable for assessing whether an aptamer-target interaction exists at all and whether its strength is compatible with downstream sensing concepts. Negative-sequence controls are a useful complementary safeguard when feasible, but they are not part of the K_d -gate pass/fail definition used in this audit. A range of solution-based methods can establish binding in homogeneous solution without target immobilization (Le et al., 2026), and this work does not prescribe a specific methodology; it asks only whether at least one appropriate homogeneous solution-phase method was used to establish binding existence and strength as required to pass the K_d gate.

Practical feasibility varies across constructs and targets. In most aptasensor studies, the sensing construct exists in free solution prior to immobilization, so a construct-matched solution-phase K_d determination is usually feasible and is the default expectation under the K_d gate. In some cases, however, a fully auditable construct-matched solution-phase K_d can be impractical not because the construct is inaccessible, but because it is difficult to deploy in the quantities, concentration regimes, or measurement formats needed to constrain K_d . For example, the sensing construct may carry multiple custom modifications that make it costly to generate the mass and replicates needed for an isotherm, or the modifications may interfere with common solution-phase readouts (e.g., by adding the background or altering the observable). In such cases, a pragmatic approach is to determine K_d for the unmodified or minimally modified sequence in homogeneous solution and then perform a low-consumption bridging experiment to test whether the sensing construct preserves binding (e.g., in competition with the unmodified sequence, or an orthogonal solution assay on the modified construct when feasible). Surface-derived apparent affinities may provide supportive context, but they should be treated as supplementary because surfaces can change K_d through crowding, avidity, and interface effects (Bueno, 2025). When a fully auditable construct-matched solution-phase K_d cannot be obtained, studies should state this limitation and still provide the strongest feasible fallback evidence package rather than implying that device response alone establishes binding. Such fallback packages may materially reduce risk, but because they do not report a construct-matched solution-phase determination of K_d , they do not pass the K_d gate as operationalized in this compliance audit.

To translate these evidence requirements into a compliance audit, we applied a binary pass-fail criterion aligned with our central question: did a study verify, as a prerequisite for aptasensor development, that the sensing construct forms a bona fide complex with the intended target in homogeneous solution with a measurable affinity? A study was counted as compliant only if it reported independent binding verification that satisfied both operational requirements of the K_d gate (Fig. 1): binding had to be demonstrated in homogeneous, non-denaturing solution, and K_d had to be determined quantitatively for a construct equivalent to the sensing construct, as defined above. Studies were scored as non-compliant whenever either requirement was not satisfied. Common reasons included treating sensor calibration or LOD-based performance metrics as evidence of binding, relying only on surface-confined assays, substituting in silico docking for experimental verification, or reporting only qualitative solution experiments or quantitative measurements for a non-equivalent construct. This criterion was applied using the full article record, including supplementary materials when available. Although the K_d gate is conceptually a pre-development safeguard, chronological sequence was not treated as an audit criterion because it generally cannot be reconstructed reliably from the publication record.

Importantly, we are not evaluating biosensor performance or platform design. Device-level controls and optimization are not included in our pass/fail compliance score, because our focus is whether the study reports passing the K_d gate. However, where applicable, we note recurring device-level limitations that can increase the risk of

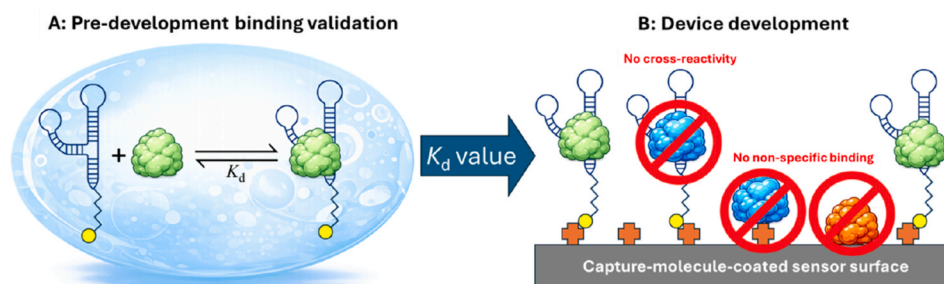


Fig. 1. Pre-development binding validation (K_d gate) and subsequent device development for the same aptasensor constructs. (A) Prior to immobilization, the aptamer construct intended for the sensor (including permanent chemical features such as linker and biotin handle) is evaluated in homogeneous, non-denaturing solution to determine an application-relevant K_d . K_d determination is performed under buffer and cosolvent conditions relevant to sensing. (B) After passing the solution-phase K_d gate, the construct is immobilized (e.g., via biotin–streptavidin coupling) and binding is re-established and verified in the device format. Negative controls exclude cross-reactivity with non-target molecules and nonspecific binding of target and non-target molecules to the capture-molecule-coated surface. Symbol key: blue strand, aptamer construct (including permanent modifications/handle); green shape, intended target; yellow circle, generic aptamer handle/label (e.g., biotin or reporter); gray bar, sensor surface; orange symbols, surface capture layer (capture molecules); crossed-out blue/orange shapes, representative non-target/background species used to illustrate cross-reactivity and nonspecific adsorption controls.

interpreting sensor response as binding in the absence of upstream verification, including the frequent absence of a key negative control: replacing the aptamer with a scrambled or otherwise validated nonbinding sequence.

3. Compliance assessment

Using the evidence requirements and binary compliance criterion defined in Section 2, we audited the aptasensor literature to determine whether studies passed the K_d gate under the operational criteria defined in this audit. Throughout, compliance statements refer only to the dataset examined here and to the operational K_d -gate criteria defined in this audit.

To keep the full-text audit tractable while remaining informative, we focused on six targets: C-reactive protein (CRP), carcinoembryonic antigen (CEA), Mucin-1 (MUC1), aflatoxin B1 (AFB1), cocaine, and ATP. These targets were selected a priori from mature aptasensor literatures. Each target is represented by at least 20 aptasensor-development publications spanning at least 10 years and multiple transduction formats and independent groups. Many targets satisfy these criteria; exhaustive coverage is not the goal here. Rather, this criterion-based set was chosen to provide a stringent cross-section of mature target literatures, rather than a statistically representative sample of the entire field, spanning distinct analyte classes and application contexts, and allowing us to test whether the K_d gate is routinely implemented and reported where construct-matched solution-phase verification should be most feasible, informative, and expected. The full list of audited publications and extracted data is provided in the Supporting Information (Dataset S1), including complete citations for all papers. Each paper was screened and scored using the workflow in Fig. 2, which operationalizes the K_d gate into two pass/fail requirements. Following peer-review feedback, we revisited the cocaine literature and incorporated one additional aptasensor paper identified by a reviewer; all corpus-level counts and target-specific summaries reported here reflect this updated audit set. Corresponding authors of all assessed articles were invited by e-mail to verify the data extracted from their papers. Fourteen authors responded; no inaccuracies were identified. We begin with CRP, a clinically central protein target with a long aptasensor literature.

3.1. CRP aptasensors – 26 papers

3.1.1. Target context and constraints

CRP is a Ca^{2+} -binding pentameric plasma protein and an established clinical biomarker of inflammation and infection (Pepys and Hirschfield, 2003; Thompson et al., 1999). The native circulating form relevant to

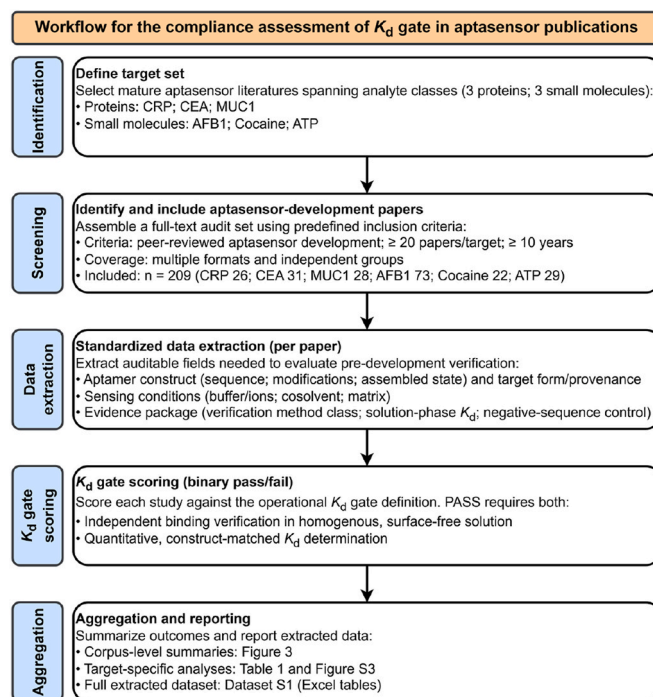


Fig. 2. Workflow for K_d -gate compliance assessment in aptasensor publications. Six representative aptasensor literatures were selected across protein and small-molecule targets, and a full-text audit set of 209 peer-reviewed development papers was assembled using predefined inclusion criteria. Standardized data extraction captured aptamer construct identity, target form and provenance, sensing conditions, and the evidence reported for pre-development verification. Each paper was then scored using a binary pass-fail criterion to determine whether it passed the K_d gate under the operational criteria described in Section 2. Aggregated results were used for corpus-level summaries, target-specific analyses, and release of the full extracted dataset.

clinical testing is Ca^{2+} -bound pentameric CRP (pCRP). Ligand recognition requires Ca^{2+} to stabilize both the binding site and the subunit interfaces (Mullenix and Mortensen, 1994), making Ca^{2+} dependence a clear biochemical indicator of whether experimental conditions preserve native structure and activity. Clinically, CRP is measured by established laboratory and point-of-care immunoassays that reliably detect native pCRP (Pohanka, 2022; Roberts, 2004).

3.1.2. Recognition element provenance and traceability

CRP aptasensor studies do not rely on a single aptamer sequence but on multiple nucleic-acid constructs with uneven traceability to the originating selection reports. Within this landscape, one DNA sequence, 5'-CGA AGG GGA TTC GAG GGG TGA TTG CGT GCT CCA TTT GGT GTT-3', has been repeatedly adopted for CRP sensing since 2010 and illustrates how provenance and validation gaps can propagate through reuse. This pattern likely reflects historical validation norms in early aptamer and aptasensor development rather than negligence in individual reports; however, repeated reuse did not resolve the underlying uncertainty about target form and independent solution-phase binding. The originating study used surface-based SELEX with CRP immobilized on magnetic beads in Ca^{2+} -containing buffer and characterized binding by SPR with immobilized CRP, reporting an apparent K_d of about 3.5 nM (Huang et al., 2010). The report lacked raw sensorgrams, replicate traces, fitting parameters, controls, an EDTA kill test for Ca^{2+} dependence, and clear specification of the biochemical form of CRP. Despite these limitations, the sequence became a widely reused recognition element in later CRP aptasensor studies.

3.1.3. Pre-development evidence gap

None of the 26 CRP aptasensor papers reported an independent, quantitative homogeneous solution-phase verification of complex formation between the aptamer construct used and native pentameric CRP in Ca^{2+} -supported solution. Only three studies provided any solution-phase evidence at all, and that evidence was qualitative rather than isotherm-based (Liu et al., 2019; Yan et al., 2023; Zhang et al., 2025). Instead, binding was typically inferred from device response or from surface- or interface-associated measurements.

3.1.4. Device-level evidence limitations

Only four of the 26 audited CRP aptasensor papers explicitly reported a scrambled or otherwise non-binding oligonucleotide control. CRP also imposes target-specific validation requirements. Across the audited studies, sensor responses were not explicitly shown to depend specifically on native Ca^{2+} -bound pCRP rather than on Ca^{2+} -depleted CRP or monomeric CRP (mCRP), and no study reported Ca^{2+} loss-and-restoration tests to connect signal to CRP's known biochemical requirement. Where selectivity was assessed, it was not designed to distinguish pCRP from plausible serum confounders, including other abundant proteins and related pentraxins. Thus, even when analytical response to CRP was reported, the evidence usually remained insufficient to show that the device was responding specifically to native pCRP recognition by the sensing construct.

3.1.5. Matrix validation and LOD interpretation

Matrix demonstration was common in the CRP aptasensor literature, typically in serum and, less often, in plasma, whole blood, or related biological samples. However, these demonstrations were usually performed under substantially modified sample conditions, including dilution, spiking, or limited pretreatment, and therefore do not by themselves establish that the sensing construct recognizes native pentameric CRP under biochemically appropriate conditions. This concern is especially important for CRP because native pCRP is Ca^{2+} -dependent, yet explicit controls showing preservation or restoration of Ca^{2+} -dependent recognition were absent. Accordingly, matrix compatibility and target-form compatibility often remained unresolved even when apparent analytical performance in biological samples appeared strong. LOD claims in the CRP literature also require cautious interpretation. The lowest reported LOD is 5 fg/mL (approximately 4.3×10^{-17} M or 0.043 fM for 115-kDa pCRP) (Vance and Sandros, 2014), which would yield an equilibrium site occupancy of about 0.012 ppm (roughly one bound CRP per ~80 million sites) if $K_d \approx 3.5$ nM were accurate. At such vanishing equilibrium occupancy, large device-level responses are difficult to attribute to equilibrium aptamer-mediated capture alone. Although amplification and device architecture can improve analytical

readout, they also propagate background, nonspecific interactions, and matrix-dependent noise; therefore, such LOD claims should not be treated as evidence of specific aptamer-CRP recognition without upstream solution-phase verification and appropriate device-level controls.

3.1.6. Compliance determination

Across the 26 CRP aptasensor papers audited here, none passed the K_d gate under our defined criteria. Specifically, no study reported an auditable, construct-matched, solution-phase determination of K_d for native pCRP under homogeneous, Ca^{2+} -supported conditions as an upstream validation step. Instead, CRP recognition was inferred from device response and other surface-associated readouts, and CRP-specific biochemical controls, such as Ca^{2+} loss and restoration, were not reported.

3.1.7. Required actions

CRP aptasensors illustrate how an unverified recognition element can become entrenched through repetition. The originating report for the repeatedly reused 2010 DNA sequence does not establish quantitative solution-phase binding to native pCRP, and across the audited CRP set the only explicitly reported solution-phase K_d evidence pertains to mCRP. Because mCRP and native pCRP are distinct target forms, evidence for binding to mCRP should not be treated as evidence for binding to native pCRP. Reported CRP aptamer candidates should therefore be retested in homogeneous solution against native pCRP in Ca^{2+} -containing buffer, with explicit target-form specification and CRP-specific biochemical controls, including Ca^{2+} loss and restoration. Only after such binding is confirmed should device optimization and analytical calibration proceed. The next example, CEA, shows similar gaps in a different biomarker class and another widely reused aptamer system.

3.2. CEA aptasensors – 31 papers

3.2.1. Target context and constraints

CEA (CEACAM5) is a heavily glycosylated cell-adhesion glycoprotein and a long-established cancer biomarker used to monitor colorectal and other carcinomas (Gold and Goldberg, 1997). Conventional detection relies on antibody-based immunoassays, often chemiluminescent (Yu et al., 2025). Aptamer sensors have been proposed as lower-cost, regenerable alternatives.

3.2.2. Recognition element provenance and traceability

Although multiple CEA recognition constructs appear in the aptasensor literature, one short DNA sequence, 5'-ATA CCA GCT TAT TCA ATT-3', is reused so frequently that it effectively functions as the de facto "CEA aptamer," often with terminal labels, spacers, or embedded in hairpins and amplification schemes. In many papers, the citation trail for this sequence does not resolve to a primary peer-reviewed SELEX report that provides both the exact sequence and quantitative binding verification under clearly specified conditions. In several cases, it instead leads to the non-peer-reviewed 2013 Smith patent application (not granted) (Smith, 2013) or to later aptasensor papers that inherit the sequence without adding independent binding verification. Patent documents can help trace sequence lineage, but they often lack the experimental detail needed to serve as primary evidence for construct-specific binding claims.

3.2.3. Pre-development evidence gap

No paper in the 31-paper CEA aptasensor set reported a solution-phase determination of K_d for the widely reused 18-mer ATA CCA GCT TAT TCA ATT as an upstream validation step. Only two studies reported any homogeneous solution-phase behavior consistent with CEA recognition by this construct, but neither reported an auditable binding isotherm or determined K_d under defined, sensing-relevant conditions; both instead relied on assay-level calibration curves for CEA

quantification (Khang et al., 2017; Wang, Y. et al., 2019). Most papers reported qualitative or semi-quantitative assay responses in which the presumed recognition event was coupled to fluorescence, photocurrent, or electrochemical signal changes, often with strong downstream amplification. Such responses do not by themselves demonstrate equilibrium complex formation in free solution or establish K_d under defined conditions. Because downstream amplification can generate robust analytical signals even when upstream molecular recognition remains weak, ambiguous, or unresolved, device-level response should not be treated as a substitute for direct solution-phase verification. Only one study outside the aptasensor-only subset reported quantitative binding analysis for this sequence or close variants using surface-based methods such as BLI, which is useful for comparing sequences but does not satisfy the requirement for homogeneous, non-denaturing, solution-phase verification under application-relevant conditions (Wang, Q.-L. et al., 2019). Independent evidence that this sequence forms a CEA-aptamer equilibrium complex in free solution under defined conditions therefore remains absent.

3.2.4. Device-level evidence limitations

Device-level rigor was similarly limited. No paper explicitly reported an aptamer-replacement control using a scrambled or validated nonbinding sequence; where mismatch controls appeared, they were used for nucleic-acid generality rather than sequence-specific CEA recognition. Selectivity testing was usually limited to a small set of non-target proteins and rarely included controls targeted to the sensing mechanism, such as surface chemistry, labels, nanomaterials, or amplification leakage. Controls isolating surface contributions were also uncommon. Thus, even when analytical response to CEA was reported, the evidence generally remained insufficient to show that the device was responding specifically to a verified CEA-aptamer interaction rather than to coupled assay behavior.

3.2.5. Matrix validation and LOD interpretation

Matrix demonstration was common in the CEA aptasensor literature, but it rarely resolved whether the sensing construct recognized a well-defined CEA form under application-relevant conditions. This concern is especially important for CEA because it is heavily glycosylated and often sourced with poorly defined provenance, preparation history, stabilizers, and glycosylation-related features. Accordingly, an aptamer optimized against one CEA preparation may not generalize to another, even when analytical signal is observed. Matrix-matched blanks, regeneration checks, and raw sensor data were also uncommon, making it difficult to separate genuine target recognition from matrix effects, surface contributions, or amplification background.

CEA LOD claims were frequently ultra-low; at least 14 papers report mass-based LODs below 1 pg/mL, including fg/mL claims, and 22 of 31 used $S/N = 3$ or 3 σ -style criteria, sometimes reporting method-dependent values within the same paper. As in CRP, impressive LOD values are not, by themselves, evidence of authentic molecular recognition, especially when target definition and independent binding verification are missing. In this context, low nominal LODs and strong responses in biological matrices should be interpreted as properties of the assay format, not as evidence that the sensing construct has been shown to bind a clearly defined CEA target in free solution.

3.2.6. Compliance determination

Across the 31 CEA aptasensor papers audited here, none passed the K_d gate under our defined criteria. Specifically, no study reported an auditable, construct-matched, solution-phase determination of K_d under defined, sensing-relevant conditions. Three recurring deficiencies were also observed: unclear or non-primary sequence provenance, incomplete control frameworks, and over-reliance on sensor signal changes as evidence of target binding in the absence of a scrambled-sequence control. Their recurrence across independent studies indicates systemic rather than incidental shortcomings.

3.2.7. Required actions

As in CRP, a recognition element can become entrenched through repetition even when independent pre-development validation is missing. The evidentiary landscape differs, however. For CRP, the limited solution-phase K_d evidence in our set pertained to mCRP rather than native pCRP, creating a target-form mismatch. For CEA, we identified some quantitative binding reports outside the aptasensor-only subset using surface-based methods such as BLI for the widely reused 18-mer and close variants, but no homogeneous solution-phase determination of K_d under clearly specified, application-relevant conditions within the CEA aptasensor set. CEA also imposes an added biochemical challenge because it is heavily glycosylated and often sourced with poorly defined provenance and form, increasing the risk that an aptamer optimized against one preparation will not generalize. Reported CEA aptamer candidates should therefore be re-tested in homogeneous, non-denaturing solution before device development, with explicit reporting of buffer composition, raw binding data, and well-defined CEA preparations, including provenance, stabilizers, and glycosylation-related attributes. Only candidates that show reproducible binding under application-relevant ionic conditions and matrix components should be advanced to device construction, after which analytical calibration and optimization can proceed. Thus, the CEA case shows that unverified sequence reuse is not confined to CRP or to any specific sensing format. The final protein-target example, MUC1, extends this pattern to peptide-selected aptamers for complex glycoproteins.

3.3. MUC1 aptasensors – 28 papers

3.3.1. Target context and constraints

MUC1 is a large, heavily glycosylated transmembrane glycoprotein overexpressed in many epithelial cancers (Nath and Mukherjee, 2014). Its tandem-repeat domain contains PDTR motifs that are commonly targeted by antibodies and aptamers (Karsten et al., 1998; Zhou et al., 2018). Clinical immunoassays detect soluble MUC1 fragments for cancer diagnosis and monitoring. Because glycosylation and target form modulate epitope accessibility, binding verification under non-denaturing, application-relevant conditions is essential for any diagnostic recognition element. For the purposes of pre-development verification, an application-relevant MUC1 form should be operationally defined and explicitly specified. At minimum, this includes whether the target is full-length MUC1, a shed extracellular fragment, or a short peptide, together with its glycosylation status, expression system, fusion tags or stabilizers, and whether it represents membrane-associated or soluble MUC1 (Hattrup and Gendler, 2008; Kufe, 2009). In the absence of such definition, affinity claims cannot be unequivocally interpreted or compared across studies.

3.3.2. Recognition element provenance and traceability

Most MUC1 aptasensors reuse a DNA aptamer first reported in 2006 (S2.2 family; 5'-GCA GTT GAT CCT TTG GAT ACC CTG G-3'), originally selected against short PDTR-containing peptides (Ferreira et al., 2006). The original report characterized binding by ELISA-type formats and SPR rather than by independent homogeneous-solution K_d determination. In our dataset, the 2006 family sequences (Ferreira et al., 2006), and close derivatives recur throughout the MUC1 aptasensor literature, often without reassessment of binding to application-relevant MUC1 forms. The implicit extrapolation from peptide epitope to intact glycosylated MUC1 remains unverified. This extrapolation presents a mechanistic uncertainty: peptide-selected aptamers may bind a linear PDTR motif in isolation but may not recognize the same motif inside the structural and glycosylation context of native MUC1. O-linked glycans in the tandem-repeat region can sterically obstruct peptide epitopes, modify local conformation, and influence multivalent presentation on the cell surface. Consequently, binding to a short synthetic peptide alone does not establish recognition of full glycosylated MUC1 under physiological conditions. The distinction is particularly important for

diagnostic applications targeting circulating or membrane-associated MUC1 species. As with CRP and CEA, repeated reuse of a canonical sequence has created an appearance of validation through citation, while leaving the required quantitative homogeneous-solution verification step systematically unaddressed.

3.3.3. Pre-development evidence gap

Across the 28 reviewed MUC1 aptasensor studies, none reported a K_d determination for binding to application-relevant MUC1 forms, such as full-length glycosylated MUC1, in homogeneous non-denaturing solution as an upstream validation step. Studies commonly used peptide epitopes, MUC1-positive cell lines, or commercial MUC1 “antigen/protein” standards, but these inputs rarely had sufficient target-form specification to support molecular-level conclusions. Two of 28 studies included solution-based qualitative evidence consistent with binding but still did not provide an independent K_d determination (Jiang et al., 2017; Tian et al., 2022).

3.3.4. Device-level evidence limitations

Selectivity was typically inferred from cross-analyte panels, peptide-competition experiments, or MUC1-positive versus MUC1-negative cellular comparisons. Sequence-level negative controls were rare: only one of 28 studies explicitly reported a scrambled or otherwise nonbinding sequence. Target-form controls (e.g., testing distinct MUC1 preparations or glycosylation dependence) were largely absent. As a result, device response is often interpreted as specific binding even when the tested target is a peptide epitope, a cell-surface surrogate, or an insufficiently specified MUC1 standard, none of which establishes binding to native glycosylated MUC1 in free solution under defined non-denaturing conditions.

3.3.5. Matrix validation and LOD interpretation

Matrix-related validation in the MUC1 aptasensor literature was limited and often difficult to interpret because the target form itself was insufficiently specified. Matrix-matched blanks, regeneration steps, and kinetic or stability checks were inconsistently reported. This is especially important for MUC1 because glycosylation, shedding state, and presentation context can strongly influence epitope accessibility. Accordingly, analytical response in biological or cell-derived settings does not by itself establish recognition of a well-defined application-relevant MUC1 form.

A second recurring concern is the plausibility of the ultra-low LODs reported for MUC1. Several studies report LODs in the 10^{-15} g/mL range, and one paper reports an LOD of 8.1×10^{-18} g/mL (signal-to-noise ratio, S/N = 3) using a 30- μ L assay aliquot (Zhao et al., 2024). At that concentration, the total mass of analyte per assay is 2.4×10^{-19} g, corresponding to fewer than one molecule on average for a 200 to 500 kDa MUC1 species (approximately 0.7 to 0.3 molecules). In this regime, Poisson sampling predicts that many replicate aliquots would contain zero target molecules, making routine reproducible analog detection and calibration-based quantification difficult to interpret. Without independently proven molecular recognition and stringent background controls, calibration linearity at such low nominal copy numbers may reflect correlated background signals, surface-driven amplification, amplification of noise, or matrix effects rather than stochastic capture of individual MUC1 molecules. Therefore, ultra-low LOD values cannot substitute for upstream homogeneous-solution binding verification and explicit target-form definition (Rissin et al., 2010).

The same concern extends, though less extremely, to multiple 10^{-15} g/mL claims that correspond to only tens to hundreds of molecules per assay volume yet are typically reported as computed LODs, such as S/N = 3 or $3 \times \text{SD (blank)}/\text{slope}$, without rigorous accounting for sampling statistics, target definition, and matrix effects. Such ultra-low LOD claims do not validate molecular recognition; instead, they highlight the need for target-form specification, independent quantitative homogeneous-solution binding verification, and stringent

background controls before device-level performance can be meaningfully interpreted. In such regimes, the apparent LOD may be dominated by correlated background, nonspecific adsorption, or amplification of matrix-dependent noise present at far higher effective abundance than the nominal target, producing apparent calibration behavior while remaining unrelated to specific MUC1 binding.

3.3.6. Compliance determination

As with CRP and CEA, none of the MUC1 aptasensor papers audited here passed the K_d gate under our defined criteria. The prevailing pattern involves constructing assays or devices from peptide-selected aptamers and inferring specificity from device-level controls. Yet no study reports an auditable, construct-matched, solution-phase K_d for an application-relevant MUC1 form under defined sensing conditions. Consequently, none passed the K_d gate. This indicates a conceptual, workflow-level omission: device development proceeds before verifying in homogeneous solution that the recognition interaction exists and is quantitatively characterized under relevant conditions.

3.3.7. Required actions

Future development of MUC1 aptasensors should begin with independent quantitative homogeneous-solution verification under physiologically relevant, non-denaturing conditions using well-defined MUC1 materials with explicit target-form and glycoform specification, together with appropriate negative, matrix, surface or interface, and regeneration controls. Peptide-selected aptamers should be explicitly re-evaluated against application-relevant MUC1 forms, and new selections should use non-denaturing conditions that preserve glycosylation and target presentation. Establishing these practices will align MUC1 aptasensor research with the foundational principles of molecular recognition and intended applications.

3.4. AFB1 aptasensors – 73 papers

3.4.1. Target context and constraints

AFB1 is a hydrophobic small-molecule mycotoxin, so both aptamer binding and sensor performance are highly sensitive to assay chemistry, including the organic cosolvents often required for solubilization and sample handling (Zhang and Banerjee, 2020). Across the 73 AFB1 aptasensor studies, cosolvent use is frequently mentioned, but the final cosolvent identity and composition under actual binding or sensing conditions are often not reported clearly enough to be auditable. The chemical form of the target used for binding or verification is also not always explicit, for example free AFB1 versus conjugated or labeled analogs, even though these forms can differ substantially in binding behavior. These are not minor reporting details: for hydrophobic small molecules, solvent composition and target form define the effective binding environment and can materially affect apparent affinity and sensor response, making reported K_d values difficult to interpret, compare, or transfer to application-relevant conditions.

3.4.2. Recognition element provenance and traceability

Across the 73 AFB1 aptasensor studies, 43 reuse a canonical DNA aptamer lineage built on the 50-nt sequence 5'-GTT GGG CAC GTG TTG TCT CTC TGT GTC TCG TGC CCT TCG CTA GGC CCA CA-3' or on direct truncations, most commonly the 47-nt variant. When a primary source is specified, the earliest disclosure and initial affinity claims for this lineage are often traced to patent literature (frequently the NeoVentures patent PCT/CA2010/001292 published in 2011) (Le et al., 2011), while many papers do not clearly identify a peer-reviewed SELEX source. Notably, rigorous solution-phase studies do exist for this AFB1 aptamer lineage, including systematic measurements of affinity and condition dependence and structural elucidation of high-affinity recognition by the 26-mer truncation AF26 using high-resolution solution NMR supported by ITC and SPR (Sun and Zhao, 2018; Xu et al., 2023). In subsequent aptasensor studies, this lineage is routinely carried into new

platforms and reformatted with sensor-specific features such as terminal labels, spacers, or immobilization handles. Because these modifications can change folding and binding behavior, citing the canonical sequence alone does not establish that the sensing construct retains homogeneous-solution binding under the assay chemistry used for detection unless construct- and condition-matched solution-phase verification is explicitly reported.

3.4.3. Pre-development evidence gap

Across the 73 AFB1 papers, binding verification remains dominated by inference from device response rather than by independent confirmation in free solution. Only 8 papers report any solution-phase verification of binding for the aptamer used (Chen et al., 2021; Dong et al., 2023; He et al., 2025; Hu et al., 2025; Li et al., 2023; Wang, C. et al., 2019; Wang, C. et al., 2020; Zhang et al., 2018). Of these, three report a numeric K_d , while five provide only qualitative solution-phase evidence. Even within the quantitative subset, two of the three reported K_d values are not interpretable because the datasets do not constrain K_d adequately (see next section).

3.4.4. Near-exceptions: what the rare numeric K_d reports show

Three papers report independent homogeneous-solution K_d determinations, with values of 32.6 nM, 435.2 ± 63.2 nM, and 16 ± 4.2 nM (the first two cases present more than two significant digits, which is hardly attainable in K_d determination) (He et al., 2025; Li et al., 2023; Wang, C. et al., 2020). However, inspection of the reported binding isotherms (Figs. S1 and S2) indicates that the first two values should not be treated as reliable verification. The 32.6 nM value is derived from a dose-response curve with poorly defined upper and lower plateaus and no measurements spanning the expected inflection region ($x \sim K_d$) (He et al., 2025), so the fitted parameter is weakly constrained by the data. The 435.2 ± 63.2 nM value is derived from an isotherm that does not extend to concentrations comparable to the reported K_d and does not reach the inflection region ($x \approx K_d$) (He et al., 2025), making K_d effectively non-identifiable and the fit dominated by a near-linear regime.

In contrast, the 16 ± 4.2 nM value is reported from ITC with stated conditions and uncertainty and good-quality binding isotherm (Wang, C. et al., 2020). This is a sequence-matched near exception in our audit: the ITC determination used the unmodified parent sequence rather than a construct matched to the sensing construct, including any terminal modifications and, where relevant, the assembled recognition state, under sensing-relevant buffer and cosolvent conditions. One additional paper reports a surface-derived estimate of K_d (≈ 21.8 nM) back-calculated from a reported surface association constant (Guo et al., 2025). We did not scrutinize this determination because it does not qualify as homogeneous-solution verification.

Importantly for this compliance audit, none of these reports explicitly establishes that K_d was determined for the same construct used in the sensor and under the same buffer and cosolvent conditions used for sensing. Taken together, these reports are best viewed as near-exceptions rather than evidence of routine compliance: two do not constrain K_d well enough for reliable determination, one provides a higher-quality sequence-matched solution-phase determination but still lacks explicit construct and condition matching, and all retain reporting gaps that make the results vulnerable to misinterpretation.

3.4.5. Device-level evidence limitations

Device-level controls do not substitute for homogeneous-solution binding verification. This is particularly relevant for AFB1 aptasensors, which often employ nanomaterials, amplification cascades, or surface gating where signal changes can arise from adsorption, matrix effects, or correlated backgrounds. Notably, only one paper explicitly reported a scrambled or validated nonbinding aptamer replacement control to test sequence-specificity. Many studies instead rely on cross-analyte selectivity panels against other mycotoxins. Ochratoxin A (OTA) is the most common comparator, tested in 56 of 73 papers,

whereas structurally related aflatoxins are assessed less consistently. Such panels can support a claim of preferential device response, but they do not establish that the sensing construct forms a specific equilibrium complex with AFB1 in homogeneous solution under the assay conditions used for detection.

3.4.6. Matrix validation and LOD interpretation

Matrix work is widespread, with both buffer and matrix validation in 39 of 73 studies and spiked-only validation in 30, but matrix performance does not replace binding verification and can amplify the risk of correlated-background artifacts. LOD claims span an extreme range. Among the 70 papers with LOD values convertible to mass concentration, the median LOD is ~ 3.2 pg/mL with an interquartile range of 0.3–37 pg/mL, and the minimum reported value is 0.9 ag/mL. Sub-pg/mL claims occur in 23 papers, including five with sub-fg/mL claims. Where detection aliquot volumes are reported (54 papers), conversion of LOD to molecule counts yields a median of 1.3×10^9 molecules at LOD with an interquartile range of 4.7×10^7 – 1.3×10^{10} and a minimum of ~ 870 molecules. Such low-copy-number regimes are inherently vulnerable to stochastic sampling, adsorption losses, correlated background, and amplification of nonspecific or matrix-dependent noise, reinforcing the need for independent solution-phase binding verification and device-level controls before performance is interpreted as evidence of specific aptamer-AFB1 recognition.

3.4.7. Compliance determination

None of the 73 AFB1 aptasensor papers passed the K_d gate under our defined criteria. The dominant pattern is device construction and performance reporting without an auditable, de novo, construct-matched solution-phase determination of K_d for the recognition construct against free AFB1 under sensing-relevant buffer and cosolvent conditions. One paper represents a sequence-matched near-exception: it reports a well-documented solution-phase ITC determination of K_d for the underlying aptamer sequence under stated conditions, but does not explicitly establish a construct-matched solution-phase K_d for the exact sensing construct under sensing-relevant conditions. Two additional papers report numeric homogeneous-solution K_d values, but the reported datasets do not constrain K_d adequately (no inflection-region coverage and/or incomplete saturation), making them unsuitable as auditable verification. A single report does include an aptamer-replacement nonbinding-sequence control, but this does not substitute for construct- and condition-matched solution-phase K_d determination (Wang and Zhao, 2020). Taken together, the rare numeric K_d reports do not establish construct- and condition-matched verification and therefore do not demonstrate routine compliance.

3.4.8. Required actions

The AFB1 case supports the same policy conclusion as the protein targets, with additional AFB1-specific requirements that directly support the K_d gate. At minimum, studies should (i) explicitly state target form in both verification and sensing, for example free AFB1 versus conjugated or labeled analogs; (ii) report the AFB1 stock solvent and the final cosolvent identity and percentage in all binding and sensing steps so that K_d determinations can be judged for relevance; (iii) perform an independent homogeneous-solution determination of K_d for the exact sensing construct under buffer and cosolvent conditions matching those used for sensing; and (iv) report key negative controls, including an aptamer-replacement control using a scrambled or validated nonbinding sequence rather than only interferent panels, which is rarely reported in the AFB1 literature (Wang and Zhao, 2020).

3.5. Cocaine aptasensors – 22 papers

3.5.1. Target context and constraints

Cocaine is a small-molecule analyte of high societal and forensic relevance. Its aptasensor literature is often presented as a model system

for structure-switching DNA constructs, signal amplification strategies, and rapid testing concepts. Unlike protein targets, cocaine is typically used as a well-defined free analyte in aqueous buffer, reducing ambiguity in target form. This simplicity should make pre-development solution-phase verification especially feasible and expected.

3.5.2. Recognition element provenance and traceability

The cocaine-binding aptamer literature draws heavily on a canonical DNA aptamer lineage first reported by Stojanovic in 2001, centered on the widely reused core sequence 5'-GAC AAG GAA AAT CCT TCA ATG AAG TGG GTC-3', and subsequently reformatted across diverse transduction modalities into sensor-specific constructs (Stojanovic et al., 2001). Across our 22-paper set, this recognition sequence appears in multiple independent reports, often with substantial engineering changes such as immobilization handles, spacers, hairpin constraints, split designs, and reporter-coupled variants. While these modifications are central to sensor operation, they also sever any evidentiary link to the binding properties of the original construct unless the modified construct is re-verified. Therefore, the relevant recognition element is not the canonical aptamer sequence as originally reported, but the specific aptamer construct actually used for sensing, which should be re-verified in homogeneous solution before assay implementation or device construction.

3.5.3. Pre-development evidence gap

In this 22-paper cocaine aptasensor set, binding is inferred predominantly from sensor behavior rather than established independently in homogeneous solution. One paper passes the K_d gate: an optical cocaine aptasensor based on a triple-fragment aptamer reports ITC-based homogeneous-solution determination of K_d for the same assembled recognition construct used for sensing, with the TFA construct (probes 3, 4, and 5) reporting $K_d = 111.2 \pm 19.1 \mu\text{M}$ under stated buffer conditions (Zou et al., 2012). The same study also reports that the TFA assay must be conducted at 4 °C, matching the temperature used for the TFA binding determination, and includes sequence-level control fragments and matrix demonstration in urine and serum. Thus, this paper shows that construct-matched homogeneous-solution verification is feasible within an engineered cocaine sensing format. One additional paper remains a near-exception rather than a pass: it reports a quantitative value from a homogeneous solution experiment, but this does not constitute a direct K_d for cocaine binding to the sensing construct (Dauphin-Ducharme et al., 2023). Instead, the reported micromolar constant is an apparent K_d derived from a competitive displacement fluorescence response using an aptamer bearing a covalently attached methylene blue reporter (MB), in which cocaine competes with MB for an intramolecular binding site and the signal reflects MB displacement or environmental change rather than a two-component binding isotherm. As a result, the fitted parameter is assay-contingent and should not be treated as homogeneous solution verification of cocaine binding for the sensing construct itself.

3.5.4. Device-level evidence limitations

Device-level calibration curves and selectivity panels are ubiquitous in cocaine aptasensors, but they do not establish the existence of an aptamer–target complex in homogeneous solution. Most studies report some interference or selectivity panel, sometimes including closely related drugs or metabolites, yet negative-sequence controls remain rare. Only five papers report a scrambled, mutated, or otherwise nonbinding sequence-level control; the remainder rely on platform-level controls, such as electrode-only baselines or omission controls, and selectivity panels. Without a nonbinding-sequence control, signal changes can arise from platform-specific mechanisms such as adsorption, ionic-strength effects, reporter interactions, surface crowding, or amplification cascades, all of which can mimic target-dependent response in the absence of genuine molecular recognition in solution.

3.5.5. Matrix validation and LOD interpretation

Fourteen of the 22 papers include spiked matrix demonstrations (e.g., diluted serum, plasma, saliva, or urine), while eight are buffer-only. LOD values span more than 10 orders of magnitude, from approximately 13 fM to 1 mM, with a median of approximately 38 nM and several sub-nM claims. Where detection aliquot volumes are reported and molecule counts can be estimated, the implied copy numbers at LOD remain reasonably large, with a median of $\sim 1.5 \times 10^{12}$ molecules with a minimum claim of 8×10^4 . Thus, the primary interpretability constraint is not low-copy-number plausibility but evidentiary linkage. In the absence of an auditable homogeneous-solution K_d determination for the construct used, even strong analytical performance cannot be unambiguously attributed to an aptamer–cocaine complex in solution. Accordingly, the solution-phase K_d reported for the triple-fragment cocaine construct demonstrates the feasibility of upstream construct-matched verification, but it should not be taken as sufficient to justify or predict the LODs of surface-confined cocaine aptasensors, whose apparent performance can be strongly altered by immobilization, probe density, and other interfacial design variables.

3.5.6. Compliance determination

Across these 22 cocaine aptasensor papers, one passed the K_d gate. The dominant workflow nevertheless remains device construction followed by inference of binding from sensor response, with limited use of negative-sequence controls and, in most cases, without an auditable, construct-matched solution-phase determination of K_d for the sensing construct under sensing-relevant conditions.

3.5.7. Required actions

The cocaine case study shows that passing the K_d gate is feasible even when the sensing construct is heavily engineered, but still remains rare despite the simplicity of the target form and the comparatively tractable solution chemistry. At minimum, cocaine aptasensor studies should (i) perform an independent homogeneous-solution determination of K_d for the exact aptamer construct used for sensing and under sensing conditions, (ii) explicitly report key solution parameters governing structure-switching constructs, including ionic strength, Mg^{2+} where used, pH, and temperature, and (iii) include a scrambled or nonbinding-sequence control alongside standard selectivity panels. Embedding these requirements as clear reporting expectations would convert sensor performance claims into interpretable evidence of molecular recognition and help prevent device-level artifacts from being misclassified as binding.

3.6. ATP aptasensors – 29 papers

3.6.1. Target context and constraints

ATP is a canonical small-molecule target in aptamer and aptasensor research and, in some studies, an intracellular metabolite monitored in living cells. ATP recognition by the canonical DNA aptamer is strongly condition-dependent, with divalent ions (especially Mg^{2+}) and ionic strength influencing aptamer folding and apparent specificity (Huizenga and Szostak, 1995; Xia et al., 2013). Accordingly, transparent reporting of ionic conditions is essential for interpreting and comparing ATP aptasensor claims; however, only 15 of 29 studies specify Mg^{2+} in the binding or assay buffer in a clear, auditable way. This ATP-binding aptamer is also reported to contain two non-identical ligand-binding sites, further reinforcing the need for clear condition reporting and explicit binding-model assumptions when affinity is assessed (Lin and Patel, 1997; Slavkovic et al., 2020).

3.6.2. Recognition element provenance and traceability

The ATP aptasensor corpus is dominated by the Huizenga-Szostak ATP-binding DNA aptamer lineage (ABA27) and its derivatives (Huizenga and Szostak, 1995). Among the 29 ATP aptasensor papers, 24 explicitly contain the ABA27 core sequence 5'-ACC TGG GGG AGT ATT

GCG GAG GAA GGT-3' within the construct used, while the remainder use split versions, constrained hairpins, or incompletely reported sequences. Across platforms, the aptamer is routinely reformatted with immobilization handles, spacers, hairpin constraints, split fragments, fluorophore-quencher pairs, and nanomaterial conjugations. These engineering changes are central to sensor operation but sever evidentiary linkage to prior binding claims unless the aptamer construct used for sensing is re-verified in homogeneous solution under sensing-relevant conditions.

3.6.3. Pre-development evidence gap

For ATP, binding is again inferred primarily from sensor response rather than established by quantitative homogeneous-solution binding studies. None of the 29 papers reports an independent, construct-matched homogeneous-solution K_d for ATP. Only 3 of 29 papers include any solution-phase re-verification, and these provide qualitative evidence rather than K_d determination (Huo et al., 2016; Srinivasan et al., 2019; Wang et al., 2016). One paper quantifies binding in homogeneous solution for thioflavin T (ThT), a fluorescent probe ligand, to the ATP aptamer ($K_d \sim 90 \mu\text{M}$), and then uses ATP-driven displacement in solution as evidence of ATP interaction (Wang et al., 2016). However, it does not determine an ATP-aptamer K_d in the study, instead citing a literature K_d value. The other two provide qualitative solution evidence consistent with ATP-dependent response but likewise do not report an ATP-aptamer K_d (Huo et al., 2016; Srinivasan et al., 2019). Thus, even the strongest near-exceptions do not satisfy the K_d gate.

3.6.4. Device-level evidence limitations

ATP aptasensors often include selectivity panels against other nucleotides, such as ADP, AMP, GTP, CTP, and UTP, which can support a claim of analytical selectivity within the device. However, such panels do not establish the existence or strength of an ATP-aptamer complex in homogeneous solution for the construct used. Negative-sequence controls are uncommon: only 5 of 29 papers report a scrambled or otherwise nonbinding control, often implemented as a mismatch, mutant, random DNA, or non-photocleavable negative construct rather than a true scrambled aptamer. Without a negative-sequence control, target-dependent signals can arise from platform-specific mechanisms such as adsorption to nanomaterials, ionic-strength effects on reporters, surface crowding, or amplification cascades, all of which can mimic binding-dependent response.

3.6.5. Matrix validation and LOD interpretation

Nineteen of the 29 papers include some matrix demonstration beyond buffer-only testing (spiked urine, serum, cell lysate, cell medium, or live-cell contexts), while 10 of 29 are buffer-only. Reported LODs span a very broad range. Among the 28 papers with concentration-type LOD units that can be expressed in molar terms, the minimum reported LOD is 1 pM, the maximum is 3.7 mM, and the median is approximately 14 nM. Where aliquot volumes were reported (12 papers), the implied molecules-at-LOD estimates span approximately 4.8×10^8 to 2.1×10^{14} molecules per assay aliquot, with a median of $\sim 1.7 \times 10^{12}$. These copy numbers do not, by themselves, validate binding. Rather, they emphasize that the primary limitation is evidentiary linkage: without independent homogeneous-solution K_d values for the construct used, analytical performance cannot be cleanly attributed to a verified molecular-recognition interaction.

3.6.6. Compliance determination

Across the 29 ATP aptasensor papers audited here, none reported an auditable, construct-matched solution-phase determination of K_d for ATP binding by the sensing construct, and thus none passed the K_d gate. The dominant workflow remains device construction followed by inference of binding from device response and selectivity panels, with limited negative-sequence controls and incomplete reporting of key ionic conditions that govern aptamer folding and apparent specificity.

3.6.7. Required actions

ATP is a chemically defined target with abundant options for homogeneous-solution binding determination, so persistent non-compliance is difficult to explain by technical infeasibility. At minimum, ATP aptasensor studies should (i) determine ATP-aptamer K_d in homogeneous solution for the exact construct used for sensing and under sensing conditions, including Mg^{2+} and ionic strength; (ii) report complete buffer composition, pH, temperature, and divalent-ion conditions for both binding verification and sensing; and (iii) include a true nonbinding-sequence control, such as a scrambled or otherwise validated nonbinder, alongside standard nucleotide selectivity panels. Embedding these requirements as clear pre-development reporting expectations would help prevent device-level artifacts from being interpreted as binding evidence and convert ATP aptasensor claims into auditable molecular-recognition evidence consistent with the core policy argument of this manuscript.

4. How inadequate characterization compromises performance

The central consequence of bypassing construct-matched solution-phase verification is not that every resulting aptasensor must necessarily perform poorly. Rather, the problem is that the molecular basis of the reported device response remains insufficiently established. In that situation, analytical performance may be real at the device level, but it cannot be attributed with confidence to a verified aptamer-target interaction under sensing-relevant conditions. As a result, performance claims can become mechanistically ambiguous, difficult to transfer, or difficult to compare meaningfully across studies.

Because an aptasensor is an integrated analytical system, observed response reflects not only molecular recognition but also the effects of surface confinement, immobilization chemistry, probe density, spacer architecture, reporter placement, local ionic conditions, target presentation, and signal-transduction architecture (Garrote et al., 2019; Leung et al., 2021; Liu et al., 2014; Rozenblum et al., 2019; Saunders et al., 2025). Accordingly, once a construct is transferred from homogeneous solution to a sensor interface, its apparent binding behavior can diverge substantially from its intrinsic solution-phase behavior. For this reason, a construct-matched solution-phase determination of K_d is a necessary upstream baseline for establishing that the sensing construct can bind the intended target, but it is not by itself sufficient to justify or predict device-level performance once that construct is immobilized or otherwise reformatted.

Following transfer from homogeneous solution to a sensing interface, several classes of design variables can alter apparent affinity and specificity, including (i) surface immobilization strategy and probe density, (ii) spacer architecture and linker length, (iii) labeling and reporter placement, and (iv) buffer composition and ionic environment. These factors can modify folding, accessibility, local concentration, and signal transduction, and therefore decouple device-level response from intrinsic solution-phase binding.

A first consequence concerns specificity and selectivity claims. When construct-matched solution-phase binding has not been independently verified, selective sensor response may reflect a combination of interfacial effects, matrix-dependent signal modulation, reporter-dependent behavior, or assay-conditional interactions rather than a well-defined recognition event (Garrote et al., 2019; Zhao et al., 2022). Under such circumstances, it becomes difficult to determine whether observed selectivity arises from intrinsic molecular recognition by the aptamer or from the specific architecture of the sensing format. This distinction matters because only the former can reasonably be expected to transfer across platforms, labels, and operating conditions.

A second consequence concerns sensitivity and limit-of-detection claims. A low limit of detection is often interpreted as evidence of strong or efficient target recognition, yet that inference is not secure unless the recognition step has been independently established. In the absence of solution-phase validation, strong analytical response at low

analyte concentration may still be achieved, but its mechanistic origin remains uncertain. Indeed, device optimization may converge on interfacial phenomena rather than improved target capture: changing probe density, immobilization scheme, spacer length, labeling strategy, or buffer composition may improve signal-to-noise, suppress drift, or alter the interfacial environment without establishing a stronger or more transferable molecular-recognition event (Garrote et al., 2019; Leung et al., 2021; Liu et al., 2014). Accordingly, steep calibration slopes or ultra-low nominal LODs should be interpreted as properties of the assay architecture unless they are anchored to independently verified upstream binding of the construct used for sensing. In this sense, inadequate aptamer characterization does not invalidate the reported analytical figures of merit outright, but it limits what those figures can be taken to mean.

A third consequence concerns reproducibility and robustness. If recognition has not been established independently of the sensing format, then changes in surface chemistry, linker length, reporter placement, target form, buffer composition, or sample matrix can alter the observed response in ways that are difficult to interpret. Performance may then appear unstable across laboratories or across later design iterations, not necessarily because the sensor is intrinsically unreliable, but because the contribution of the underlying aptamer-target interaction has never been cleanly separated from format-dependent effects. Independent solution-phase verification provides an anchor for diagnosing such behavior by distinguishing loss of genuine binding from perturbation introduced during sensor integration (Leung et al., 2021; Liu et al., 2014).

This issue becomes especially important for transferability. Across the audited literatures, the same canonical aptamer lineages were often reused across substantially different assay architectures, target preparations, and sample environments. Without construct-matched solution-phase verification under relevant conditions, it is difficult to justify transferring performance expectations from one format to another, from one target form to another, or from buffer studies to more complex matrices. The resulting problem is not only uncertainty about absolute affinity, but uncertainty about whether two studies are interrogating the same recognition event in any meaningful sense.

The same logic affects benchmarking and comparability. Device-level metrics such as response magnitude, dynamic range, and limit of detection are frequently compared across reports as though they reflect a shared molecular input. However, when the underlying aptamer construct has not been validated in solution under sensing-relevant conditions, those comparisons can become misleading. Studies may appear to benchmark the same aptamer against the same target while in practice differing in construct identity, target form, or assay-conditioned binding environment. This weakens the interpretability of cross-study performance tables and limits the field's ability to identify genuinely superior sensor designs.

Importantly, solution-phase validation is not only a safeguard against misinterpretation. It is also constructive for sensor design. Comparing the behavior of an aptamer in homogeneous solution with its behavior after integration into a sensing platform can reveal whether loss or gain of signal is associated with surface effects, reporter effects, steric constraints, target-presentation effects, or transduction-related perturbations. In that role, pre-development validation can guide optimization of sequence truncation, labeling strategy, immobilization scheme, interface composition, and assay conditions. Thus, the K_d gate is useful not only as a reporting requirement, but also as a practical tool for rational aptasensor design.

These considerations clarify why the omission of construct-matched solution-phase verification has consequences that extend beyond individual studies. The cross-target synthesis presented next shows that these vulnerabilities recur across multiple target classes and sensing formats, indicating a field-wide evidentiary pattern rather than isolated shortcomings.

5. Cross-target synthesis and policy implications

The cross-target comparison summarized in Table 1 reveals a common evidentiary pattern across six mature aptasensor literatures spanning three protein targets and three small-molecule targets. Despite substantial differences in target biochemistry, assay architecture, and application context, aptasensor development commonly proceeded without independent quantitative verification that the sensing construct forms an equilibrium complex with the intended target in homogeneous, non-denaturing solution under application-relevant conditions. In every literature examined, binding was most often inferred from device response or other format-contingent evidence rather than established directly for the construct used in sensing. At the same time, the specific vulnerabilities differed by target class. For proteins, the main recurring risks included target-form ambiguity, incomplete sequence provenance, and uncertain transfer from simplified or poorly defined recognition contexts to application-relevant analytes. For small molecules, the recurrent risks more often involved construct reformatting, condition sensitivity, and incomplete reporting of solution parameters that can strongly influence folding and apparent binding behavior.

At the corpus level, the audit indicates a stepwise erosion of evidentiary strength across the recommended pre-development workflow, as summarized in Fig. 3 and S3. Solution-phase verification was uncommon across the 209-paper dataset, and even within that limited subset the evidence was predominantly qualitative or response-based. Integrating these observations into the K_d gate shows that only one paper in the dataset passed. Importantly, that single passing study occurred in the cocaine literature and involved a heavily engineered triple-fragment sensing construct, showing that construct-matched homogeneous-solution verification is feasible even in a reformatted aptasensor design. At the same time, the rarity of such examples underscores how unusual auditable compliance remains across the broader literature. The few additional reports that presented numeric solution-phase affinity values still failed on audit because the reported constants were weakly constrained, format-contingent, and/or not construct- and condition-matched.

Taken together, these cross-target patterns point to a field-wide weakness in evidentiary practice rather than to isolated shortcomings within particular target literatures. Across diverse targets and sensing formats, the same pre-development omission recurred: device-level behavior was commonly interpreted without auditable construct-matched solution-phase verification of the sensing construct. This synthesis provides the basis for the conclusions drawn below and for the practical recommendations proposed for strengthening pre-development verification in future aptasensor work.

6. Conclusions

This focused audit of six mature aptasensor literatures shows that pre-development, construct-matched solution-phase verification remains exceptionally uncommon in aptasensor development. Across 209 audited papers, only 20 reported any solution-phase verification assay, and only 1 passed the K_d gate under the operational criteria defined here. Thus, although recommendations for upstream solution-phase validation have been articulated repeatedly in reviews and methodological discussions, they have not, in practice, functioned as an effective gate for aptasensor development.

The central implication is not that aptasensors lacking such evidence must necessarily be analytically nonfunctional. Rather, when the sensing construct has not been shown to bind the intended target in homogeneous, non-denaturing solution under relevant conditions, device-level performance cannot be attributed cleanly to verified molecular recognition. As a result, reported claims of sensitivity, selectivity, transferability, and rational optimization often rest on an uncertain mechanistic foundation.

This study was intentionally designed as a stringent cross-section of

Table 1

Cross-target summary of canonical aptamer lineage reuse, binding-verification patterns, context-specific risks, and pre-development K_d -gate outcomes in representative aptasensor literatures.

Target	Number of papers audited	Canonical lineage reused	Main binding “verification” pattern	Key context risks	Papers passing the K_d Gate
CRP	26	2010 CRP DNA aptamer lineage from surface-based SELEX against immobilized CRP (Huang et al., 2010)	Mostly device-level inference; rare solution-phase evidence, when present, is qualitative or response-based only	Target-form ambiguity (pCRP vs mCRP), strict Ca^{2+} dependence, and buffer compatibility with native pCRP	0
CEA	31	2013 patent-derived 18-mer lineage functioning as the de facto CEA aptamer (Smith, 2013)	Mostly device-level inference; rare solution-phase evidence, when present, is qualitative or response-based only	Poorly defined target provenance/form and glycosylation-related variability across CEA preparations	0
MUC1	28	2006 S2.2 family originally selected against PDTR-containing MUC1 peptide motifs (Ferreira et al., 2006)	Mostly device-level inference; rare solution-phase evidence, when present, is qualitative or response-based only	Peptide-to-full-target extrapolation, glycosylation dependence, and target-form ambiguity	0
AFB1	73	2011 NeoVentures patent-derived canonical AFB1 aptamer lineage and truncation family (Le et al., 2011)	Mostly device-level inference; rare solution-phase reverification includes qualitative/response-based evidence and a few reported K_d values	Final cosolvent composition often unclear and target form may differ between free AFB1 and analog/conjugate formats	0
Cocaine	22	2001 cocaine DNA aptamer lineage, widely reused in engineered formats (Stojanovic et al., 2001)	Mostly device-level inference; one study reports a construct-matched homogeneous-solution K_d for a triple-fragment cocaine aptamer, whereas the remaining solution-based evidence is absent or assay-contingent	Extensive construct reformatting and sensitivity of structure-switching behavior to solution conditions	1
ATP	29	1995 ATP-binding DNA aptamer lineage (ABA27), widely reused in engineered formats (Huizenga and Szostak, 1995)	Mostly device-level inference; rare solution-phase evidence, when present, is qualitative or response-based only	Strong dependence on Mg^{2+} , ionic strength, folding state, and binding-model assumptions	0

six mature target literatures rather than as an exhaustive census of the entire aptasensor field. Even within that limited scope, however, the same evidentiary omission recurred across diverse target classes and sensing formats, supporting the conclusion that the problem is systemic rather than incidental. Accordingly, passing the K_d gate should be treated as a practical minimum requirement for documenting pre-development aptamer validation in aptasensor research.

7. Future perspectives

A practical next step for the field is to move from informal encouragement of pre-development validation to explicit, auditable reporting expectations. In our view, this does not require a burdensome new publication format. Rather, it can be implemented through a short submission checklist and a concise binding-verification package. At minimum, the checklist should capture the exact sensing construct, including permanent modifications and assembled recognition state where relevant; the target form and provenance; the binding and sensing conditions; the solution-phase dataset supporting the reported K_d ; and, where full construct-matched K_d determination is not feasible, an explicit statement of limitation together with the strongest feasible fallback evidence. Such a framework would allow authors, reviewers, and editors to determine quickly whether the sensing construct has been verified in homogeneous, non-denaturing solution under relevant conditions, and would make claims of upstream molecular recognition more transparent and reproducible. This checklist is intentionally limited to auditable pre-development binding verification and is not meant to replace later-stage optimization of surface immobilization, spacer design, labeling strategy, or assay buffer, which remain device-specific engineering variables after the K_d gate has been passed.

Future work should also recognize that full construct-matched solution-phase determination of K_d may not always be straightforward for heavily modified or multipart sensing constructs. In such cases, studies should state the limitation explicitly and provide the strongest feasible fallback evidence, for example, quantitative verification of the minimally modified sequence together with a low-consumption bridging experiment that tests whether the sensing construct preserves binding. These fallback packages can reduce risk and improve interpretability, but they should not be presented as equivalent to passing the K_d gate

unless they include a construct-matched solution-phase determination of K_d . Adopting this distinction would help preserve rigor while allowing practical flexibility across target classes and assay formats.

More broadly, integrating the K_d gate into manuscript submission and peer review could help shift aptasensor development toward a more cumulative and mechanistically interpretable research culture. When construct-matched solution-phase verification is documented explicitly, later studies can compare sensor architectures against a shared and auditable molecular-recognition baseline, rather than against device behavior alone. In this way, the K_d gate is not only a safeguard against misinterpretation, but also a practical foundation for more transferable benchmarking, more rational optimization, and more reliable progress in aptasensor development.

CRedit authorship contribution statement

Sergey N. Krylov: Conceptualization, Funding acquisition, Project administration, Visualization, Writing – original draft, Writing – review & editing. **Svetlana M. Krylova:** Conceptualization, Investigation, Methodology. **An Thi Hoai Le:** Formal analysis, Validation, Visualization. **Philip E. Johnson:** Investigation, Writing – review & editing. **Ryan Hili:** Writing – review & editing. **Derek J. Wilson:** Writing – review & editing. **S. Brian Kim:** Writing – review & editing. **Gwendolyn M. Stovall:** Writing – review & editing. **Guobao Xu:** Methodology, Writing – review & editing. **Boris B. Dzantiev:** Writing – review & editing. **Maria DeRosa:** Writing – review & editing. **Paulo R. Bueno:** Writing – original draft, Writing – review & editing. **Philippe Dauphin-Ducharme:** Writing – review & editing. **Julian A. Tanner:** Writing – review & editing. **William L. Whitehouse:** Writing – review & editing. **Qiang Zhao:** Writing – review & editing. **Jun-Jie Zhu:** Writing – review & editing. **Anine Crous:** Writing – review & editing. **Elena Ferapontova:** Methodology, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known financial, professional, or personal conflicts of interest that could have influenced the work presented in this manuscript. No competing interests, such as financial relationships, consulting engagements, intellectual property, or other

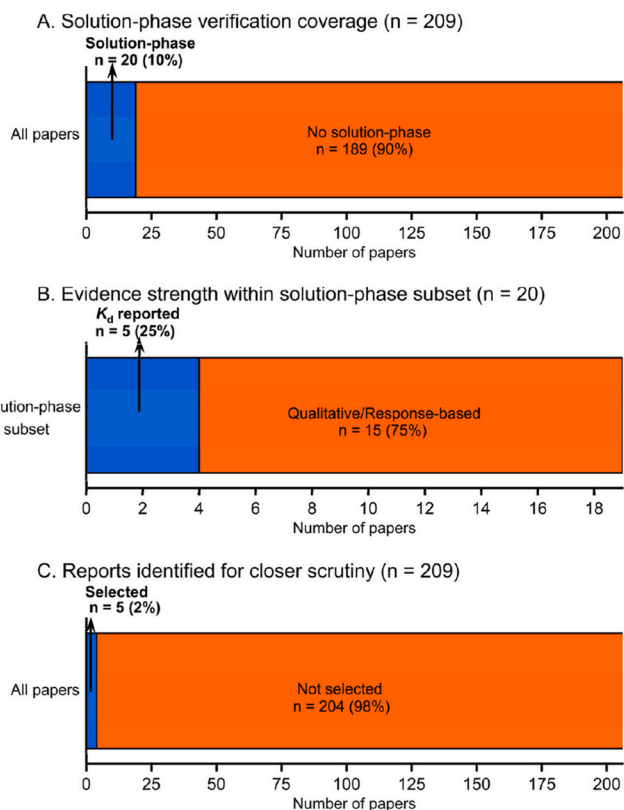


Fig. 3. Literature-wide audit of solution-phase verification across six aptasensor targets ($n = 209$). (A) Solution-based verification coverage, defined as reporting any surface-free assay intended to support target recognition independent of the sensor. (B) Evidence strength within the solution-based subset, separating reports that present a numeric K_d from those providing qualitative or response-based evidence only. (C) Reports identified for closer scrutiny, defined as reports presenting a numeric, surface-free affinity determination for the sensing construct or a closely related variant under conditions resembling those used for sensing. Among the reports counted here, one cocaine aptasensor study passes the K_d gate under the audit criteria applied in this study, whereas the remainder are near-exceptions that fail because the reported constants are weakly constrained, format-contingent, and/or not construct- and condition-matched.

affiliations, have impacted the design, execution, or interpretation of this study.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bios.2026.118807>.

Data availability

All the data are provided as supplementary information.

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SUPPORTING INFORMATION

Widespread Omission of Aptamer-Target Binding Verification in Aptasensor Development: Consequences for Sensor Performance and the Need for a K_d Gate

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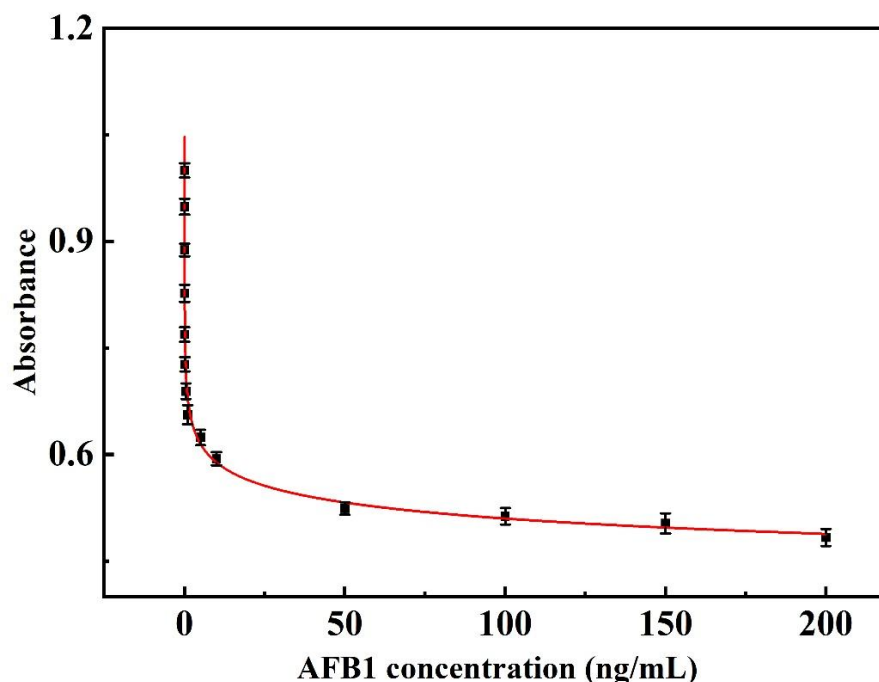


Figure S1. A binding isotherm adopted from Figure S6 in *Talanta* 287 (2025) 127620

(<https://doi.org/10.1016/j.talanta.2025.127620>) with permission:

<https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=7e43c7a4-5078-4481-81fa-02012f67df82>

The information is too dense for the first 10 points to digitize and present in a semi-log graph to observe a classic sigmoidal binding isotherm.

If the claimed value is $K_d = 32.6$ nM, then for AFB1 (MW ≈ 312 g/mol) that corresponds to about 10 ng/mL:

$$32.6 \times 10^{-9} \text{ mol/L} \times 312 \text{ g/mol} \approx 1.0 \times 10^{-5} \text{ g/L} = 10 \text{ } \mu\text{g/L} = 10 \text{ ng/mL}$$

To justify $K_d \approx 10$ ng/mL from a 1:1 binding isotherm, the data must show (or at least bracket) the inflection / midpoint region around ~ 10 ng/mL, because that is where K_d is constrained.

This plot cannot support that, for three reasons:

1. **No sampling near $x \approx K_d$.** The curve has many points compressed at ~ 0 –5 ng/mL and then jumps to 50 ng/mL. There are no data between ~ 5 and 50 ng/mL, so the critical ~ 10 ng/mL region is completely missing. Any fit that returns 32.6 nM is therefore weakly constrained (model-dependent).
2. **The transition appears to occur well below 10 ng/mL**
By ~ 5 ng/mL the absorbance has already dropped close to the “transition” zone, and by 50 ng/mL it is near the lower plateau. If K_d were ~ 10 ng/mL, you would expect the midpoint to be around 10 ng/mL and the curve to still show substantial change between, say, 5 and 20 ng/mL. This plot does not demonstrate that.
3. **The upper plateau (baseline at zero analyte) is ill-defined**
The “near-zero” points span a wide absorbance range, so the top plateau (and thus the midpoint) is not well determined. If the plateaus are uncertain, K_d becomes even less identifiable.

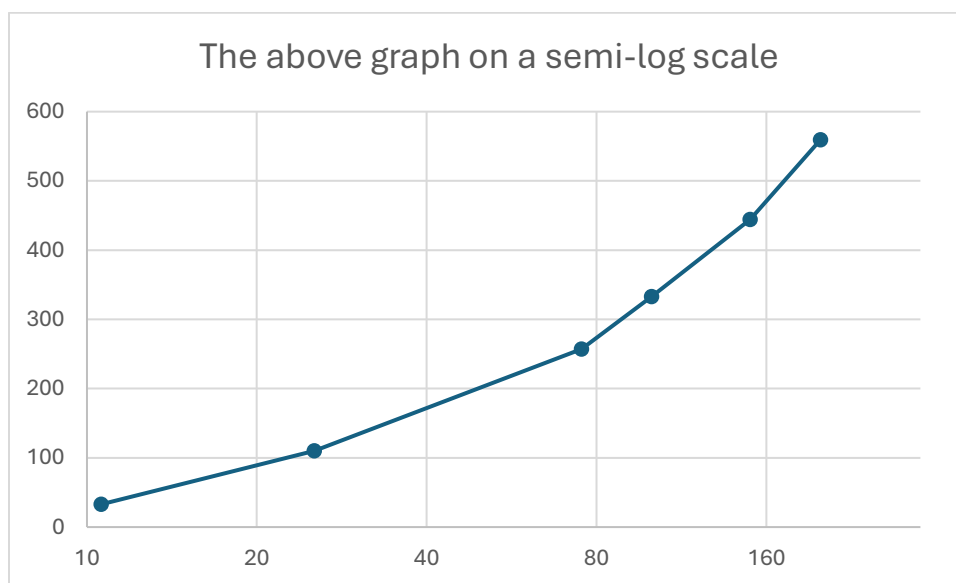
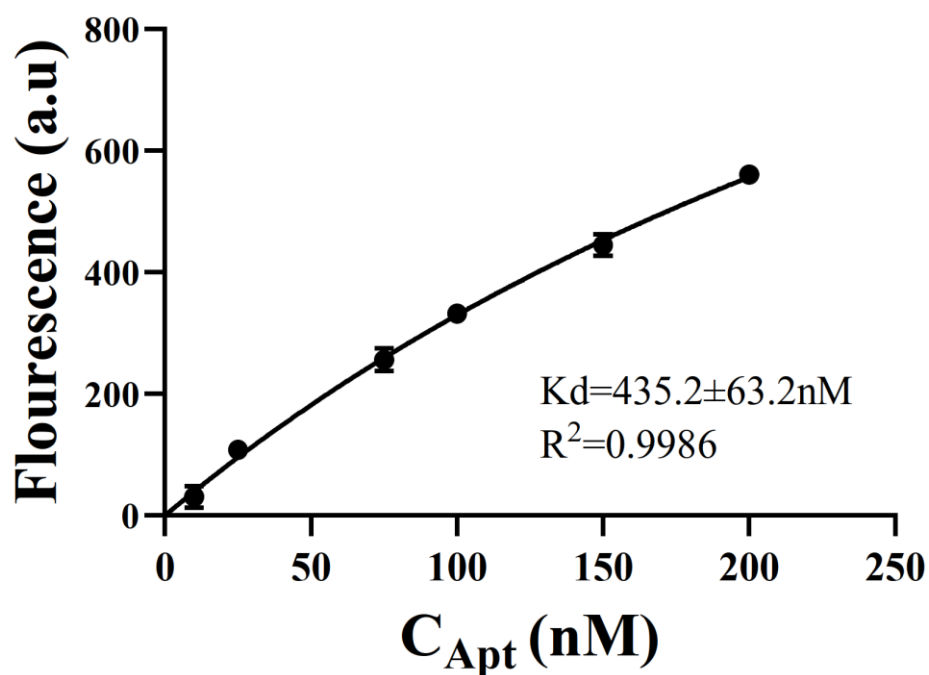


Figure S2. A binding isotherm adopted from Figure S4 in *Analytica Chimica Acta* 1279 (2023) 341844 <https://doi.org/10.1016/j.aca.2023.341844> (top) (permission: <https://s100.copyright.com/CustomerAdmin/PLF.jsp?ref=45d18ea6-4549-45df-9666-e9be70dbdfd5>) and its reconstruction in a semi-log graph (bottom)

This binding isotherm weakly constrains K_d . For a 1:1 binding isotherm written in the standard form

$$F(C) = F_0 + (F_{\text{max}} - F_0) \frac{C}{K_d + C}$$

K_d is constrained primarily by data spanning the inflection region ($C \sim K_d$) together with at least partial definition of the upper plateau ($F_{\max}$) and baseline (F_0). The dataset in Figure S2 does not meet these requirements, so K_d is effectively weakly constrained and strongly model-dependent.

(1) The concentration range does not reach the inflection region ($C \sim K_d$). The highest concentration shown is 200 nM, which is less than half of the reported K_d ($200/435 = 0.46$). If K_d truly equals 435 nM, then even at the highest point the expected fractional progress toward saturation is only

$$\frac{C}{K_d + C} = \frac{200}{435 + 200} = 0.315,$$

not reaching the inflection point of 0.5. Thus, the experiment would be sampling only the low-occupancy regime where the isotherm is close to linear and does not contain enough curvature to uniquely determine K_d .

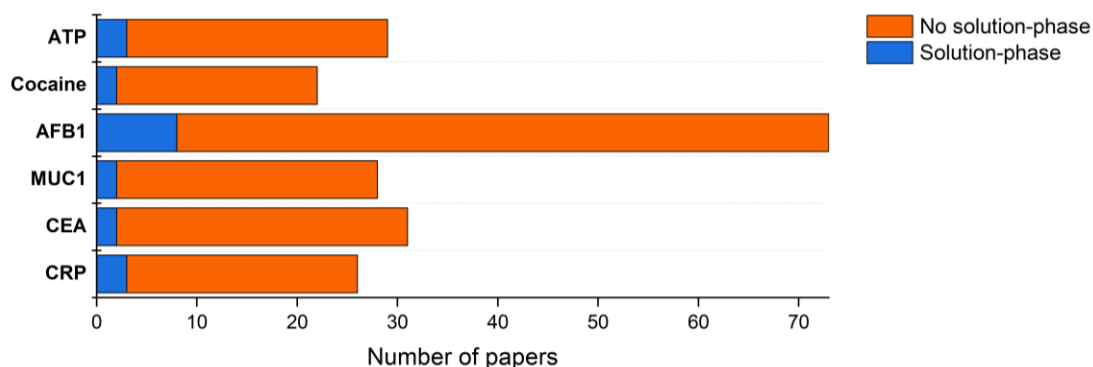
(2) Lack of an observed plateau makes K_d depend on extrapolated $F_{\max}$. Because the curve does not approach saturation, $F_{\max}$ is not determined by the data and must be inferred by extrapolation. In a 1:1 model, this creates strong parameter tradeoff between K_d and $(F_{\max} - F_0)$: many combinations of K_d and amplitude can reproduce the same six points. In fact, fitting the digitized points from Figure S2 to the model above yields a best-fit K_d near 440 nM, but the implied saturation level is far beyond the measured range ($F_{\max}$ approximately 1.8×10^3 a.u., more than 3-fold higher than the maximum observed signal near 5.6×10^2 a.u.). This demonstrates that the reported K_d relies on extrapolation to an unobserved plateau.

(3) K_d is non-identifiable in practice: wide K_d ranges fit almost equally well. Using the digitized points from Figure S2 and the same 1:1 functional form, comparably good fits ($R^2 > 0.99$) are obtained for a broad range of fixed K_d values, because the dataset never reaches the region that discriminates among them. A simple profile analysis (refitting F_0 and $F_{\max}$ at each fixed K_d) shows that K_d values spanning roughly an order of magnitude from 2.2×10^2 to 1.7×10^3 nM are statistically compatible with the data at the 95% level. This is inconsistent with the small reported uncertainty (± 63.2 nM) and indicates that K_d is not well determined from the reported titration.

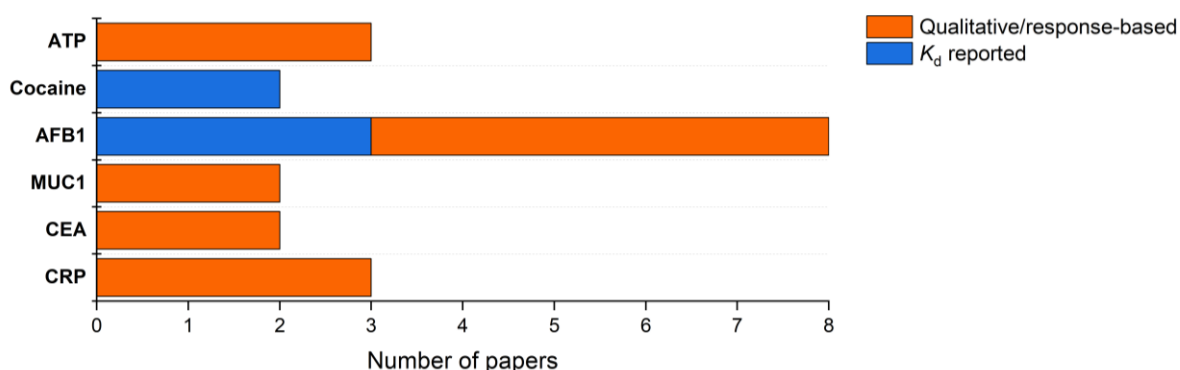
(4) Reported high R^2 does not validate K_d . A high R^2 is expected whenever a monotonic curve is fit to six monotonic points over a limited range. In the present case, R^2 mainly reflects that fluorescence increases smoothly with C_{Apt} , not that the specific parameter K_d is uniquely determined.

Implication for compliance. Because the titration does not sample C_{Apt} values near K_d and does not establish $F_{\max}$, the reported $K_d = 435.2$ nM should not be treated as reliable quantitative homogeneous-solution verification. At minimum, a defensible K_d determination would require measurements that extend to concentrations comparable to and exceeding the inferred K_d (ideally covering approximately $0.1 \times K_d$ to $10 \times K_d$) to capture the inflection region and at least approach saturation, with clear reporting of construct identity and buffer/cosolvent conditions used.

A. Solution-phase verification coverage



B. Evidence strength within solution-phase subset



C. Reports identified for closer scrutiny

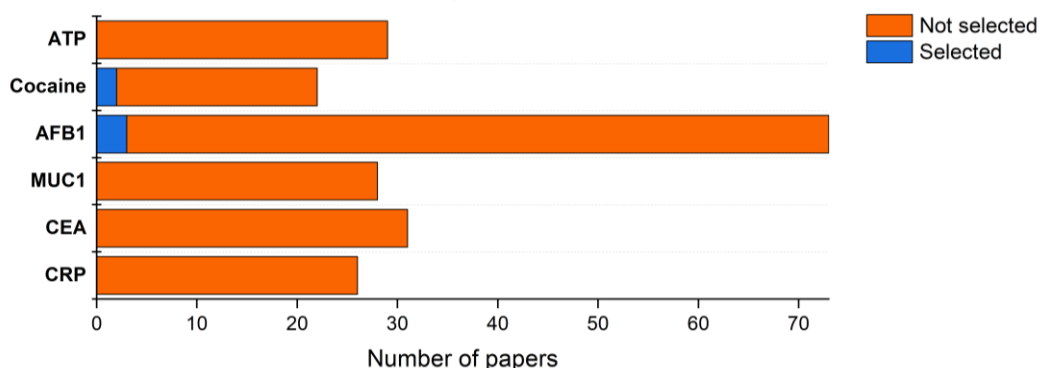


Figure S3. Literature-wide audit of solution-phase verification across six aptasensor targets ($n = 209$). (A) Solution-phase verification coverage, defined as reporting any surface-free assay intended to support target recognition independent of the sensor. (B) Evidence strength within the solution-phase subset, separating reports that present a numeric K_d from those providing qualitative or response-based evidence only. (C) Reports identified for closer scrutiny against the pre-development criterion, defined as reports presenting a numeric, surface-free affinity determination for the sensing construct or a closely related variant under conditions resembling those used for sensing. The reports counted here are mostly near-exceptions with only one passing the K_d gate under the audit criteria applied in this study.

K_d Gate Submission Checklist for Editors and Reviewers

Purpose: Confirm whether the manuscript passes the K_d gate: an auditable, construct-matched determination of K_d in homogeneous, non-denaturing solution under sensing-relevant conditions. For each item, authors should point to where it is documented (Main text section, Figure/Table, SI page, Dataset ID).

Important note: Passing the K_d gate establishes auditable upstream evidence that the sensing construct can bind the intended target in homogeneous, non-denaturing solution under relevant conditions. It does **not** by itself justify or predict device-level performance, including apparent affinity, specificity, or limit of detection (LOD), after immobilization or other sensor-specific reformatting.

A. Required to pass the K_d gate

1. Sensing construct defined (construct match)

Check all that apply:

- ☐ Full nucleic-acid sequence reported (exact length)
- ☐ All permanent chemical modifications reported (labels, linkers/spacers, terminal groups, redox tags, biotin/thiol, etc.)
- ☐ If multipart or assembled (split, duplex, structure-switching, constrained assembly): strand identities, stoichiometry, and recognition state used during binding are reported
- ☐ Explicit statement provided that the construct used for solution-phase verification is identical to the sensing construct, excluding immobilizing supports and transducer materials

Location: _____

2. Target identity, provenance, and form

Check all that apply:

- ☐ Target source/provenance reported (supplier, expression/purification context, or preparation history, as applicable)
- ☐ Chemical or biological form used in *verification* reported
- ☐ Chemical or biological form used in *sensing* reported
- ☐ Relevant target-associated features disclosed where applicable (for example: tags, labels, conjugates, stabilizers, glycoform/state, aggregation state, carrier/conjugate context)
- ☐ Explicit statement provided that the target form used for verification is the same as, or a justified surrogate for, the target form used in sensing

Location: _____

3. Solution conditions fully disclosed and sensing-relevant

Check all that apply:

- ☐ Buffer composition and pH reported
- ☐ Ionic strength and key ions reported, including divalent ions where relevant
- ☐ Additives, cosolvents (final identity and percentage), temperature, and equilibration time reported
- ☐ Final concentrations/percentages after all mixing steps are reported
- ☐ Verification conditions and sensing conditions are explicitly cross-referenced or shown side-by-side
- ☐ If verification conditions do not match sensing conditions exactly, the differences are stated and justified
- ☐ If matrix or surrogate matrix is relevant to sensing, this is stated and justified

Location: _____

4. Quantitative solution-phase K_d dataset provided for the sensing construct

Check all that apply:

- ☐ Verification method is homogeneous, surface-free, and non-denaturing
- ☐ Raw or minimally processed solution-phase binding data are provided for the construct-matched sensing construct
- ☐ Concentration coverage is sufficient to constrain K_d , including the inflection region and approach to plateau, or an explicit justification is provided if not feasible
- ☐ Reported numeric constant reflects the construct-target interaction itself, not only an indirect, assay-contingent, reporter-displacement, or device-level parameter

Location: _____

5. Auditable modeling and reporting

Check all that apply:

- ☐ Equilibrium model stated, including equation and assumptions
- ☐ Fitting approach described
- ☐ Reported K_d with uncertainty, and other fitted parameters where relevant
- ☐ Fit/reporting details are sufficient for an external reader to reproduce the reported K_d (for example: fit settings, constraints, weighting, exclusions if any)
- ☐ Any limitations in interpretability are stated explicitly (for example: indirect readout, weakly constrained fit, condition mismatch, construct mismatch)

Location: _____

6. Explicit K_d -gate matching statement

Check all that apply:

- ☐ Authors explicitly state whether the verified construct is identical to the sensing construct
- ☐ Authors explicitly state whether the verification conditions match the sensing conditions
- ☐ If either construct or conditions are not fully matched, the differences are described and the manuscript does not claim that the K_d gate has been passed on that basis

Location: _____

B. Required reporting safeguards (not part of K_d -gate pass)**7. Negative-sequence control (sequence-specificity)**

- ☐ Scrambled, mutated, or otherwise validated nonbinding sequence tested under the same solution conditions
- ☐ If feasible, the same or analogous negative-sequence control also tested in the device format

Location: _____

8. Device-level nonspecific controls (when relevant)

- ☐ Controls for nonspecific adsorption/background on the sensing interface or support material, for target and key interferents where relevant

Location: _____

C. Optional (when feasible)**9. Orthogonal confirmation**

- ☐ A second, orthogonal solution-phase method confirms the interaction and supports the reported K_d
- ☐ If not done, a brief rationale is provided

Location: _____

REVIEW OUTCOMES **K_d gate outcome (A only):**

- ☐ Passes K_d gate
- ☐ Near-exception
Numeric solution-phase affinity reported, but not fully auditable for one or more of the following reasons:
 - ☐ Not construct-matched
 - ☐ Not condition-matched
 - ☐ Target-form mismatch or insufficient target definition
 - ☐ Dataset too weakly constrained to support auditable K_d
 - ☐ Indirect or assay-contingent parameter rather than direct construct-target K_d
 - ☐ Not homogeneous, not surface-free, and/or not non-denaturing
 - ☐ Other: _____
- ☐ Does not pass K_d gate
Missing required A items: _____

Safeguards summary (B only)

- ☐ All B items reported
- ☐ Some B items reported
- ☐ No B items reported

Orthogonal confirmation (C, optional):

- ☐ Reported
- ☐ Not reported
- ☐ Not feasible / not applicable (stated)

Notes (optional): _____