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Solution-Phase ITC Validation of Literature-Reported Glyphosate DNA Aptamers: Affinity Ranking and an Operational Selectivity Boundary

Jingchun Sun [1,2,3], Linbing Zhang [2,3,4], David Gonçalves [5,6], Shaoping Kuang [1,*] and Hongsheng Yang [2,3,4,*]

1 College of Environment and Safety Engineering, Qingdao University of Science and Technology, Qingdao 266042, China
2 Laboratory of Marine Ecology and Environmental Sciences, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266000, China
3 Laboratory for Marine Ecology and Environmental Science, Qingdao Marine Science and Technology Center, Qingdao 266237, China
4 State Key Laboratory of Breeding Biotechnology and Sustainable Aquaculture, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266000, China
5 Institute of Science and Environment, University of Saint Joseph, Macau SAR, China
6 William James Center for Research, ISPA—Instituto Universitário, 1149-041 Lisbon, Portugal
* Correspondence: kuangshaoping@126.com (S.K.); hshyang@qdio.ac.cn (H.Y.)

Abstract

Glyphosate is a highly polar herbicide, the reliable molecular recognition of which is complicated by co-occurring structural analogues, metabolites, and derivatives in real-world samples. Rather than reporting new aptamer discovery, this study establishes a standardized, solution-phase isothermal titration calorimetry (ITC) workflow to thermodynamically reassess two literature-reported glyphosate DNA aptamers, Seq03 and Seq05, under matched buffer composition and instrument settings. After verification of baseline stability and evaluation of heat-of-dilution contributions, ligand-to-aptamer titrations yielded apparent dissociation constants of approximately 8.14 μM for Seq03 and 40.2 μM for Seq05, enabling affinity-based prioritization of these reported candidates within the tested concentration window. To define an application-relevant selectivity boundary, we further constructed a counter-screen panel restricted to glyphosate-related chemicals, including structural analogues, metabolites, and derivatives, and evaluated all candidates using an identical ITC protocol with explicit background handling. None of the counter-screen compounds produced binding-consistent, saturable isotherms after integration and control-based interpretation; instead, their responses remained close to background heat and were therefore operationally classified as having no detectable binding under the tested conditions, including a reverse-titration format check with Glufosinate-N-acetyl. Collectively, these results position ITC as a label-free, platform-independent validation step for small-molecule aptamer benchmarking prior to analytical translation, while also highlighting that the present conclusions are bounded by the tested PBS-based conditions and the sensitivity window of the current ITC configuration.

Keywords: glyphosate; DNA aptamer; isothermal titration calorimetry; solution-phase validation; thermodynamic reassessment; affinity ranking; counter-screening; selectivity boundary

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1. Introduction

Glyphosate (N-(phosphonomethyl)glycine) is among the most widely used herbicides worldwide, and its extensive application continues to drive demand for reliable monitoring across environmental, agricultural, and food-related matrices [1,2]. In practice, glyphosate may co-occur with structurally related analogues and transformation products, including aminomethylphosphonic acid (AMPA) and glufosinate-related compounds, thereby increasing the risk of analytical cross-reactivity and underscoring the need for recognition systems with clearly defined selectivity boundaries [3]. Although chromatographic methods remain the reference approaches for quantitative determination, the analysis of highly polar compounds such as glyphosate and AMPA often requires derivatization and matrix-intensive sample preparation, which motivates complementary molecular recognition strategies for rapid verification and selective screening when analytical specificity can be demonstrated [3,4]. Aptamers are single-stranded oligonucleotides identified through SELEX and have become programmable recognition elements for a wide range of analytical applications [5,6]. With advances in sequencing-assisted selection and optimization, aptamer discovery and evaluation have continued to mature, and aptamer-based sensing has expanded across electrochemical, optical, and related analytical formats [7–9]. However, the development of small-molecule aptamers remains particularly challenging because apparent affinity and selectivity can be strongly influenced by assay format, immobilization geometry, labeling chemistry, buffer composition, and data-analysis strategy [10,11]. As a result, selectivity-oriented design principles and screening strategies such as Capture-SELEX are increasingly emphasized to improve practical performance and reduce false positives in downstream analytical applications [12,13]. For glyphosate, several DNA aptamer-based recognition and sensing studies have been reported, including graphene oxide-assisted selection followed by label-free impedimetric detection, as well as more recent electrochemical, fluorescent/qPCR, and chemiluminescence platforms [14–17]. These studies support the analytical potential of glyphosate-binding aptamers, but they also show that reported performance is often embedded within specific sensor architectures rather than isolated solution-phase binding measurements. In particular, the candidates examined in the present work are literature-reported aptamers with different sequence lengths and likely different folding behaviors; accordingly, our goal is not to isolate sequence-length effects per se, but to benchmark representative reported candidates under matched experimental conditions. Because sensor readouts may be influenced by surface effects, interfacial electrostatics, labeling chemistry, and assay-specific transduction mechanisms, they do not necessarily provide a direct measure of intrinsic solution-phase affinity or selectivity [10,11]. Therefore, a label-free validation step performed in solution is valuable for ranking literature-reported candidates and defining selectivity boundaries against the most plausible glyphosate-related interferents prior to analytical translation. Isothermal titration calorimetry (ITC) is a label-free, in-solution technique that directly measures interaction heats and can provide binding stoichiometry (n), equilibrium dissociation constants (K_d), enthalpy (ΔH), and derived thermodynamic parameters from a single experiment [18–20]. Quantitative ITC analysis, however, depends on rigorous experimental design and data treatment, including stable baselines, appropriate injection strategy, and careful attention to concentration uncertainty and fit identifiability [21,22]. Methodological advances such as automated peak-shape analysis and integration [23,24], uncertainty evaluation frameworks [25], variance/error-structure modeling [26,27], and open tools for reproducible fitting [28] have further strengthened ITC as a platform for binding validation and cross-study comparability. These characteristics make ITC particularly suitable for a workflow in which literature-reported aptamer candidates are reassessed under matched conditions and interpreted with explicit reporting boundaries. In this study, we do not seek to report

new glyphosate aptamer discovery; instead, we apply a standardized ITC-based workflow under matched buffer composition and instrument settings to thermodynamically reassess two literature-reported glyphosate DNA aptamers, Seq03 and Seq05, and prioritize these candidates based on solution-phase affinity under the tested conditions. To define an application-relevant selectivity boundary, we constructed a focused counter-screen panel restricted to glyphosate-related chemicals, including structural analogues, metabolites, and derivatives, and evaluated all candidates using the same ITC workflow and explicit background controls. This design allows us to distinguish interpretable binding from near-background, non-saturable responses within the sensitivity and concentration window of the present PBS-based ITC configuration.

2. Materials and Methods

2.1. DNA Aptamers and Working Solutions

Two literature-reported DNA aptamer candidates for glyphosate recognition were evaluated in this study: Seq03 (60-mer) and Seq05 (36-mer). These two sequences were selected as representative reported candidates for thermodynamic reassessment under matched ITC conditions, rather than to isolate sequence-length effects. The sequences used were as follows:

Seq03 (60 nt): GGACAGCTGGCCGCGTAGCGAGACACGTACAAGGTACTATACG-GCTGGCATATGTATCTG;

Seq05 (36 nt): AGCTTGCTGCAGCGATTCTTGATCGCCACAGAGCT.

The oligonucleotides were commercially synthesized according to the published sequences by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) and were purified by high-performance liquid chromatography (HPLC). Lyophilized oligonucleotides were dissolved in phosphate-buffered saline (PBS, pH 7.4) to prepare stock solutions and were subsequently diluted to the working concentrations used for ITC measurements. Unless otherwise stated, aptamers were used at 20 μM in the sample cell for standard ligand-to-aptamer titrations. PBS was used as a standardized matched buffer condition for all measurements in the present workflow; this choice was intended to support direct cross-candidate comparison under identical experimental conditions rather than sequence-specific buffer optimization for each aptamer.

2.2. Isothermal Titration Calorimetry Workflow

All ITC experiments were performed using a MicroCal PEAQ-ITC instrument (Malvern Panalytical, Westborough, MA, USA) with PBS (pH 7.4) as the assay buffer. In the present study, PBS was employed as a standardized matched buffer condition for all measurements to enable direct comparison among aptamer candidates and counter-screen compounds within a single workflow; it was not intended as a sequence-specific buffer optimization condition for each aptamer. Prior to each run, the sample cell and syringe were cleaned using the instrument cleaning program, and instrument performance was checked by a water–water titration.

Unless otherwise specified, titrations were conducted at 25 °C with a reference power of 5 μcal/s, feedback set to "High", and a stirring speed of 750 rpm. The initial delay was 60 s. A total of 19 injections were applied at 120 s intervals, consisting of an initial 0.5 μL injection followed by 18 injections of 1.0 μL each (hereafter referred to as the standard injection protocol). Independent solution preparations were measured in triplicate (n = 3) unless otherwise stated, and representative thermograms are shown in the main figures unless noted otherwise.

2.3. Baseline and Heat-of-Dilution Controls

To quantify dilution and background heat contributions under matched conditions, PBS was loaded into the sample cell and glyphosate (1 mM) was loaded into the syringe and titrated using the standard injection protocol. These measurements were used to evaluate the magnitude and consistency of non-binding heat signals arising from ligand dilution and buffer-related background effects. Where appropriate, the resulting heats were used as background references for subsequent interpretation and subtraction. In addition, the observed background-level heat range served as an operational reference when distinguishing binding-consistent responses from near-background, non-saturable traces in later analyses.

2.4. Ligand-to-Aptamer Titrations

For binding measurements, the aptamer (Seq03 or Seq05) was loaded into the sample cell at 20 μM (0.02 mM), and glyphosate was loaded into the syringe at 1 mM. This concentration window was selected to balance instrumental sensitivity, practical sample consumption, and the relatively small heat signals typically associated with small-molecule/aptamer interactions in ITC. In particular, the aptamer concentration in the cell was chosen to maintain measurable responses while avoiding excessive sample use, whereas the ligand concentration in the syringe was selected to provide sufficient molar excess within the standard injection program. Titrations were carried out using the standard injection protocol described above.

2.5. Counter-Screen Titrations

To define an application-relevant selectivity boundary, a counter-screening panel restricted to glyphosate-related chemicals was evaluated. The panel was intentionally focused on plausible glyphosate-related interferents, including structural analogues, metabolites, salts, and derivatives, rather than chemically unrelated compounds. Each counter-screen compound was titrated under the same matched conditions used for glyphosate measurements (compound in syringe; 20 μM aptamer in cell; PBS, pH 7.4; standard injection protocol). The tested glyphosate-related chemicals are listed in Table 1.

Table 1. ITC-based counter-screening panel and operational selectivity-boundary assessment for Seq03 and Seq05 against glyphosate-related compounds under matched conditions.

No.	Compound (Syringe Unless Noted)	Category/Relation to Glyphosate	Key Functional Motifs (Qualitative)	Seq03 Heat Flow (μcal/s)	Heat Level	Seq05 Heat Flow (μcal/s)	Heat Level	Interpretation Under Tested Conditions
1	Glyphosate (1 mM)	Target	phosphonate + amine + carboxyl	~0.30	High	~0.28	High	Binding detected; K_d ~8.14 μM (Seq03), ~40.2 μM (Seq05)
2	Glycine (1 mM)	Structural analogue	amine + carboxyl	~0.015	Very low	~0.005	Very low	No detectable binding
3	Sarcosine (1 mM)	Analogue	tertiary amine + carboxyl	~0.012	Very low	~0.02	Low	No detectable binding
4	Glufosinate (1 mM)	Related herbicide	phosphinate	~0.01	Very low	~0.015	Very low	No detectable binding
5	Glufosinate ammonium (1 mM)	Related herbicide (salt)	phosphinate	~0.04	Low	~0.035	Low	No detectable binding
6	Methyl glyphosate (1 mM)	Derivative	modified/esterified glyphosate	~0.06	Moderate	~0.035	Low	No detectable binding
7	AMPA (1 mM)	Major metabolite	phosphonate + amine	~0.02	Low	~0.025	Low	No detectable binding
8	Isotope-labeled glyphosate (1 mM)	Isotope analogue	same motifs (isotope-labeled)	~0.03	Low	~0.004	Very low	No detectable binding
9	Anecortave desacetate (1 mM)	Panel compound (as used)	multi-functional	~0.02	Low	~0.02	Low	No detectable binding

Table 1. *Cont.*

No.	Compound (Syringe Unless Noted)	Category/Relation to Glyphosate	Key Functional Motifs (Qualitative)	Seq03 Heat Flow (µcal/s)	Heat Level	Seq05 Heat Flow (µcal/s)	Heat Level	Interpretation Under Tested Conditions
10	N-acetyl glyphosate (1 mM)	Derivative	acetylated amine	~0.02	Low	~0.02	Low	No detectable binding
11	PEP (1 mM)	Pathway-related analogue	phosphate-containing	~0.05	Low	~0.03	Low	No detectable binding
12	Glufosinate-N-acetyl (reverse titration)	Related derivative	acetylated	~0.05	Low	~0.01	Very low	No detectable binding (reverse format)

All measurements were performed under matched ITC settings (PBS, pH 7.4; 25 °C; 20 µM aptamer in cell; 1 mM compound in syringe unless noted; standard injection protocol). Heat-flow values are approximate peak magnitudes read from thermograms. Because counter-screen traces did not yield binding-consistent, saturable isotherms and exhibited signals close to dilution/background contributions (typically $\leq$ 0.06 µcal/s), outcomes were operationally reported as "no detectable binding under the tested conditions" rather than being over-interpreted as weak binding. Heat level bins were used for reporting convenience (e.g., very low $\leq$ 0.02; low 0.02–0.06; moderate $\geq$ 0.06; high $\geq$ 0.20 µcal/s).

2.6. Aptamer Controls and Reverse Titration

To assess systematic heats associated with aptamer injection, aptamer-to-buffer control titrations were performed by loading PBS into the sample cell and aptamer (Seq03 or Seq05) at 100 µM into the syringe, followed by the standard injection protocol.

For the reverse-titration format check, Glufosinate-N-acetyl was loaded into the sample cell at 5.6 µM (0.0056 mM), and aptamer (Seq03 or Seq05) was loaded into the syringe at 100 µM, followed by the standard injection protocol. These experiments were designed as an internal format-control assessment to determine whether titration direction influenced near-background responses for this representative counter-screen compound. They were not intended to serve as the primary basis for thermodynamic parameter extraction, but rather to support interpretation of near-background behavior under the same instrumental platform.

2.7. Data Processing, Model Fitting, and Reporting Criteria

Raw thermograms were baseline-corrected and integrated using the MicroCal PEAQ-ITC analysis software (version v1.41; Malvern Panalytical, Westborough, MA, USA). Where appropriate, heats derived from ligand-to-buffer controls were subtracted for background correction. Binding isotherms were fitted using a single-site binding model to obtain stoichiometry (n), equilibrium dissociation constant (K_d), and binding enthalpy (ΔH); ΔG and $-T\Delta S$ were calculated from standard thermodynamic relationships.

To align with reporting expectations for analytical validation, fit interpretability was evaluated based on (i) residual inspection, (ii) parameter plausibility, (iii) saturation behavior, and (iv) consistency with the observed background-level heat range and replicate measurements. Fits were considered interpretable only when the integrated heats produced a binding-consistent and saturable trend together with non-systematic residuals and physically plausible fitted values. By contrast, traces that remained near background and did not show binding-consistent saturation were not over-fitted as weak binding; instead, they were operationally reported as "no detectable binding under the tested conditions." In this study, this wording indicates that no reliably interpretable binding could be resolved within the sensitivity and concentration window of the present ITC configuration, rather than implying the absolute absence of any molecular interaction.

3. Results

3.1. Instrument Performance and Baseline Stability

Prior to the binding measurements, instrument performance was assessed by a water–water titration. The baseline remained stable throughout the measurement window (DP ~4–6 µcal/s; H < 0.05 µcal/s), indicating low drift and low background heat under the experimental settings used in this study (Figure 1). This stable baseline provided the analytical basis for subsequent interpretation of the relatively small heat signals observed in ligand-binding and counter-screen experiments.

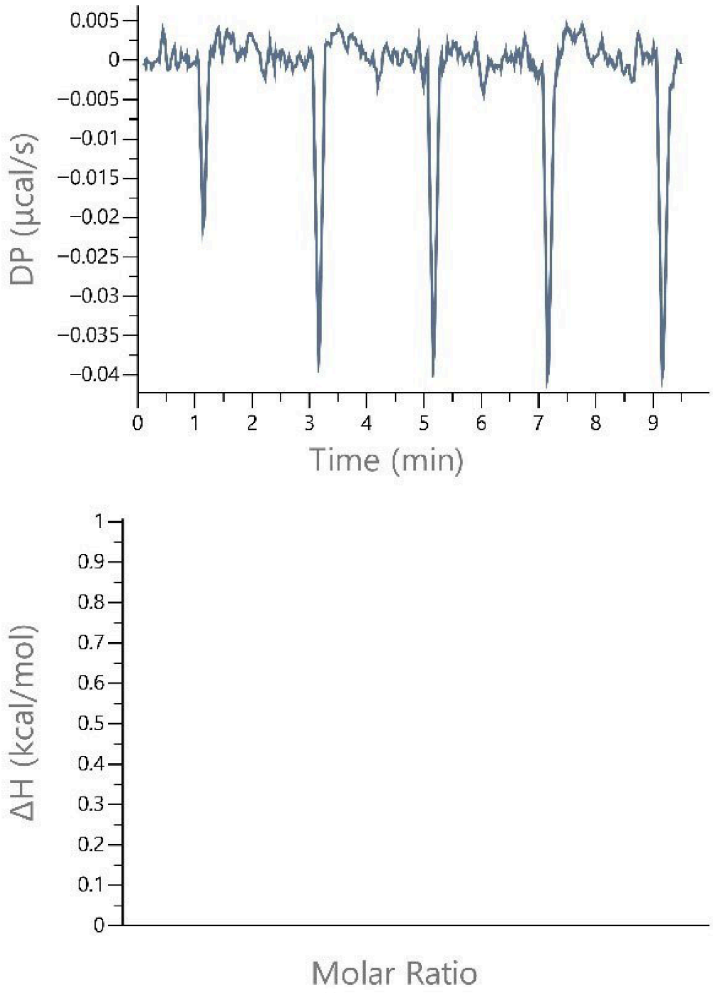


Figure 1. Water–water titration for instrument performance assessment prior to binding measurements. Stable DP and low H values indicate minimal baseline drift and low background heat under the experimental settings used in this study (DP and H are baseline diagnostics reported by the MicroCal PEAQ-ITC software: differential power and heat-flow readout, respectively).

3.2. Glyphosate Binding to Aptamers Seq03 and Seq05

Under the matched ITC conditions used in this study, both Seq03 and Seq05 produced binding-consistent calorimetric responses upon glyphosate titration. Representative thermograms obtained by titrating glyphosate (1 mM, syringe) into aptamer solutions (20 µM, cell) are shown in Figure 2. The peak heat-flow magnitude was approximately 0.30 µcal/s for Seq03 and 0.28 µcal/s for Seq05.

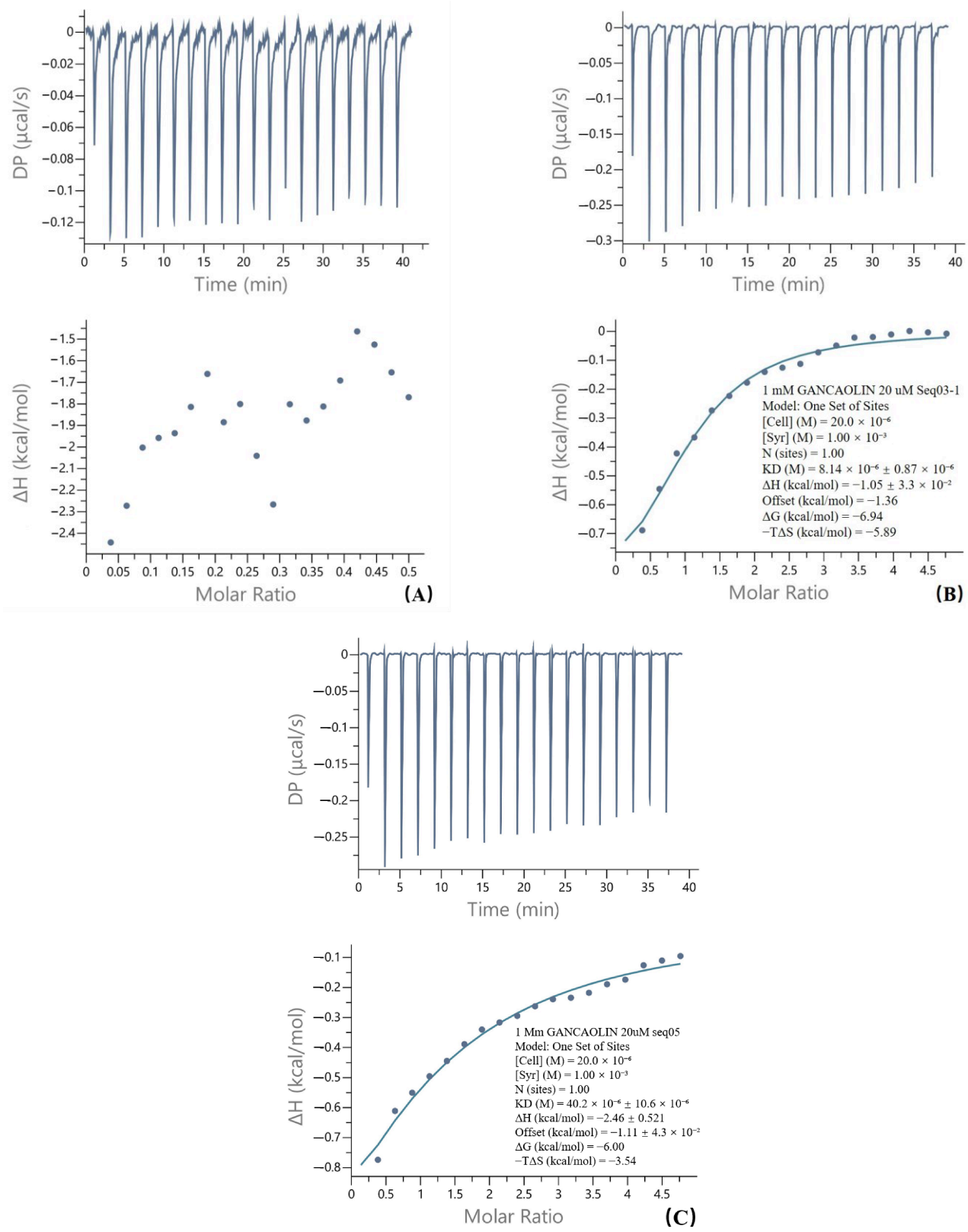


Figure 2. Representative ITC thermograms and integrated binding isotherms for glyphosate titrated into (**A**) buffer (heat-of-dilution control), (**B**) Seq03 (20 µM in cell), and (**C**) Seq05 (20 µM in cell). Glyphosate concentration in the syringe was 1 mM. Integrated heats were fitted using a single-site binding model for datasets showing binding-consistent and interpretable behavior under the tested conditions.

Fitting of the integrated heats using the single-site model yielded apparent K_d values of approximately 8.14 μM for Seq03 and 40.2 μM for Seq05. Within the tested PBS-based concentration window, these interpretable fits indicate a stronger apparent solution-phase binding response for Seq03 than for Seq05. Because the two candidates differ in sequence composition and length, this result should be interpreted as a matched-condition benchmark between literature-reported aptamers rather than as an isolated test of sequence-length effects.

3.3. Counter-Screening Defines an Operational Selectivity Boundary

To define an application-relevant selectivity boundary, counter-screen titrations were performed using a panel intentionally restricted to glyphosate-related chemicals, including structural analogues, metabolites, salts, and derivatives (Table 1), under the same ITC settings used for glyphosate measurements (compound in syringe; 20 μM aptamer in cell; PBS, pH 7.4; standard injection protocol). For both Seq03 and Seq05, none of the counter-screen candidates generated binding-consistent, saturable isotherms after integration and baseline handling (Figure 3 and Table 1). Instead, the observed heat-flow responses remained close to dilution and background contributions (typically $\leq$ 0.06 μcal/s) and did not support a reliable single-site fitting within the sensitivity and concentration window of the present configuration.

Accordingly, these near-background responses were not over-interpreted as weak binding but were operationally reported as "no detectable binding under the tested conditions." Within the present workflow, this result defines an operational selectivity boundary against the most plausible glyphosate-related interferents rather than an exhaustive specificity map across unrelated chemicals.

3.4. Reverse Titration Provides a Format Check Within the Same Platform

To further evaluate whether titration direction could influence near-background responses for a representative counter-screen compound, reverse titrations were performed using Glufosinate-N-acetyl (5.6 μM in cell) and aptamer (100 μM in syringe). Under this format, both aptamers produced only small heat signals (Seq03: ~0.05 μcal/s; Seq05: ~0.01 μcal/s) and did not generate binding-consistent, saturable behavior (Figure 4). Although the absolute heat-flow magnitudes differed between the Seq03 and Seq05 reverse-titration traces, both responses remained within the near-background regime and did not support a reliable thermodynamic interpretation.

These differences are more plausibly attributed to sequence-dependent injection/dilution effects, nucleotide composition or conformation-related background behavior, and the inherent sensitivity limits of near-background ITC measurements, rather than to selective recognition of Glufosinate-N-acetyl. Accordingly, the reverse-titration results serve as a format check within the same platform and are consistent with the counter-screen conclusion of no detectable binding under the tested conditions.

3.5. Consolidated Validation Outcomes

Across the matched ITC conditions used in this study, glyphosate produced interpretable binding thermograms for both aptamers, enabling a practical affinity-based prioritization in which Seq03 showed a lower apparent K_d and a stronger apparent calorimetric response than Seq05 under the tested conditions. In contrast, all glyphosate-related counter-screen compounds yielded near-background heat signals and non-saturable behavior and were therefore operationally classified as having no detectable binding under the tested conditions. Taken together, Table 1 summarizes the two principal outputs of the present workflow: (i) matched-condition affinity ranking for candidate prioritization and (ii) an operational selectivity boundary against the most plausible glyphosate-related interferents.

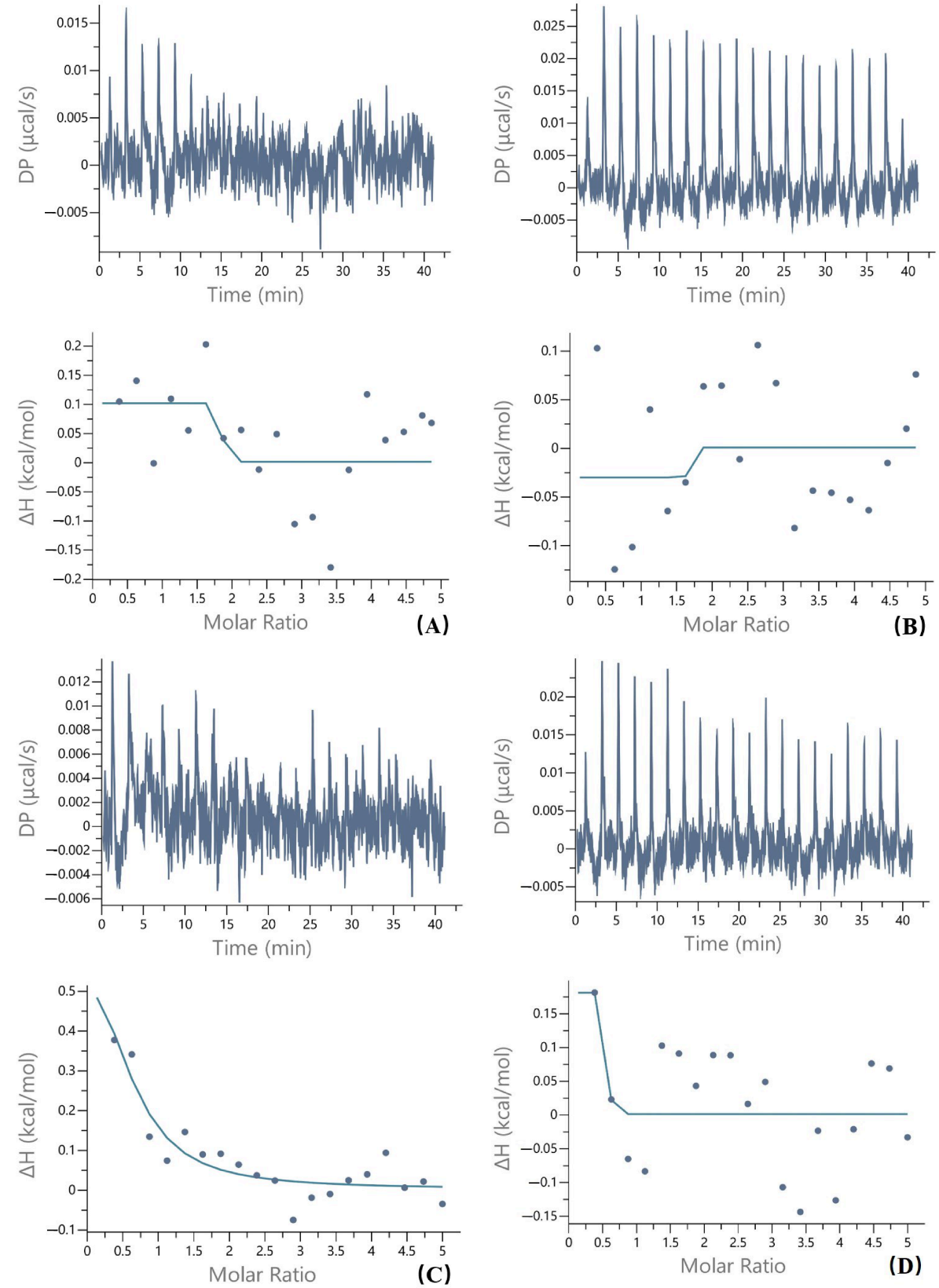
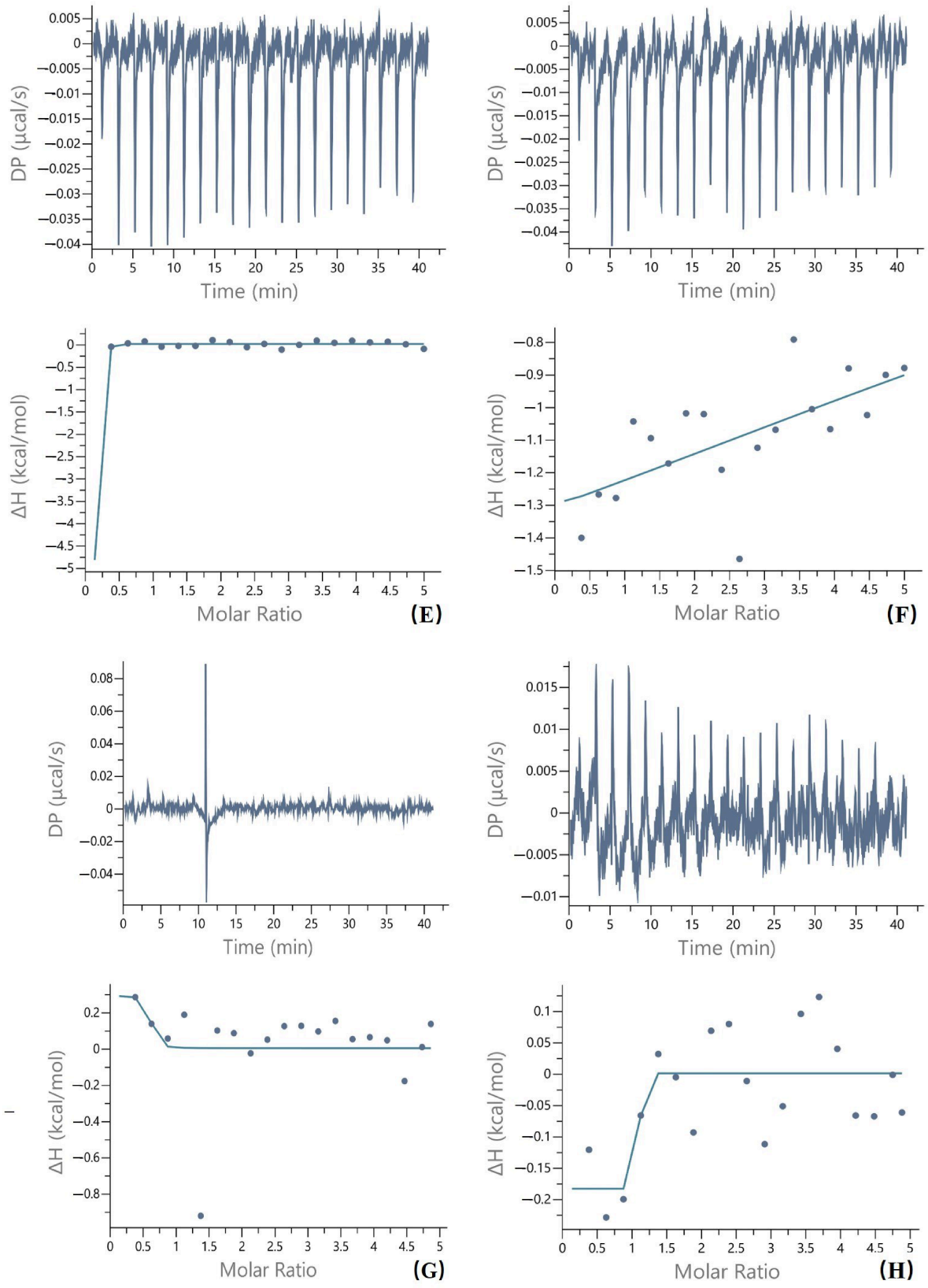


Figure 3. *Cont*.



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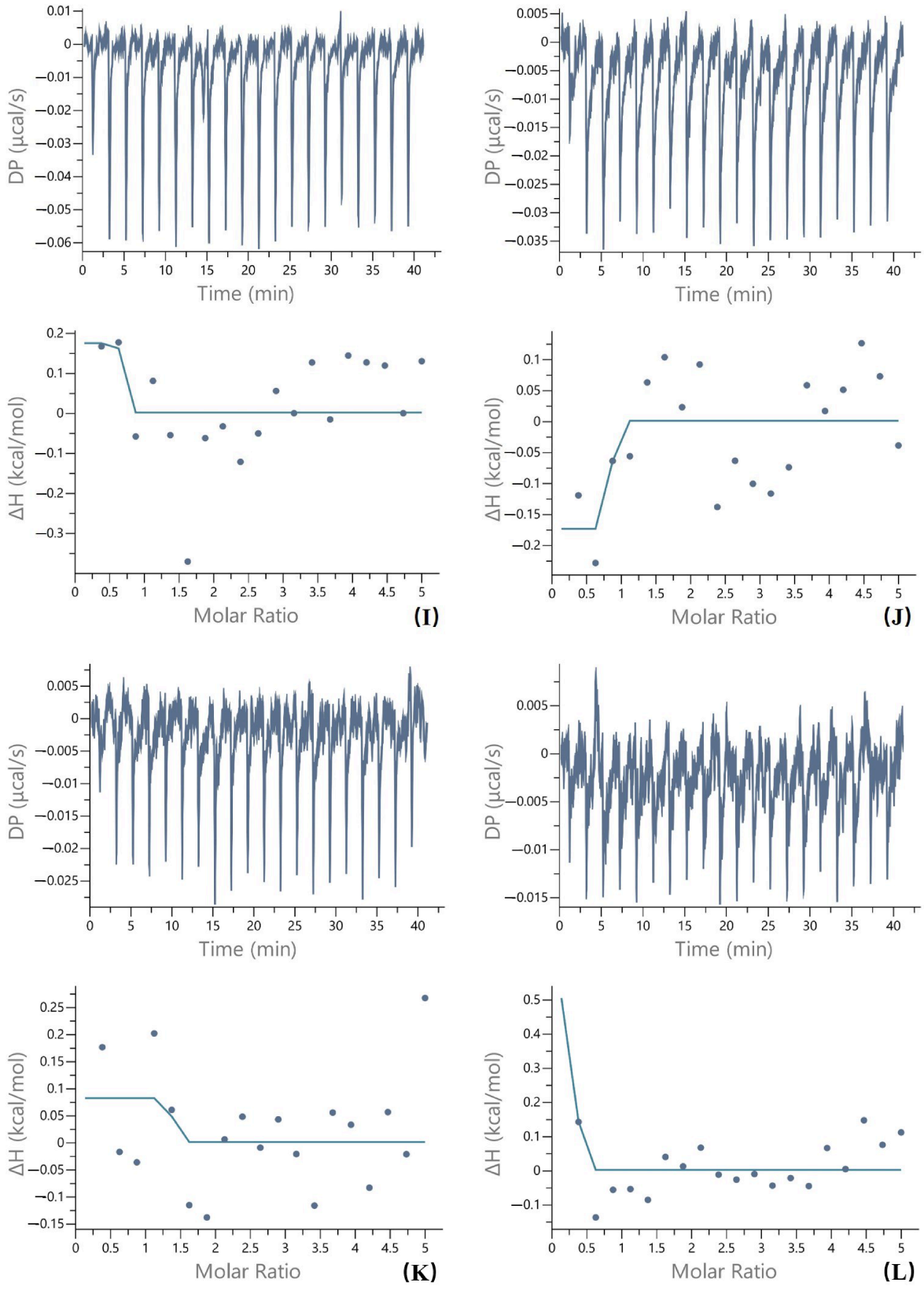

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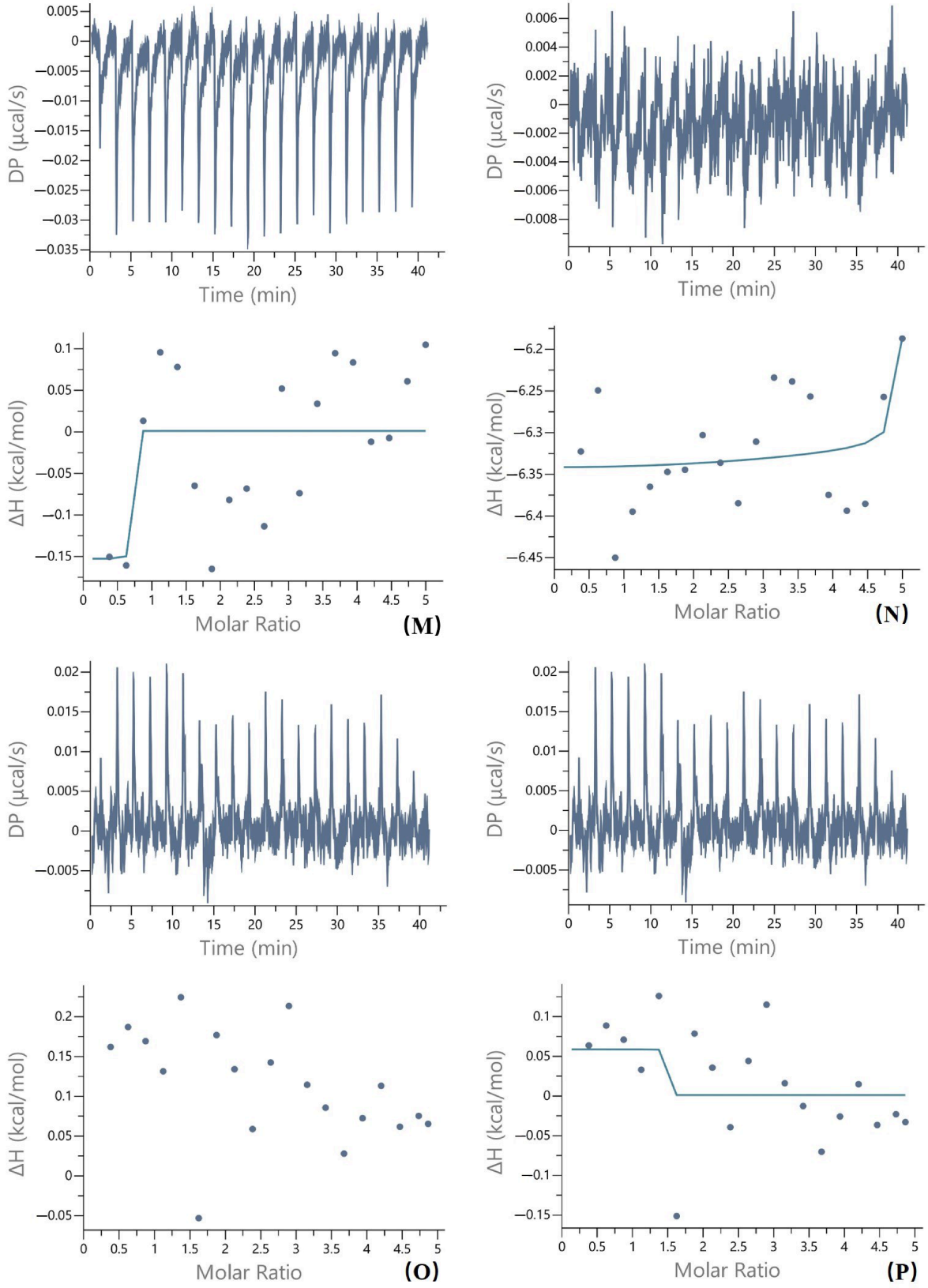

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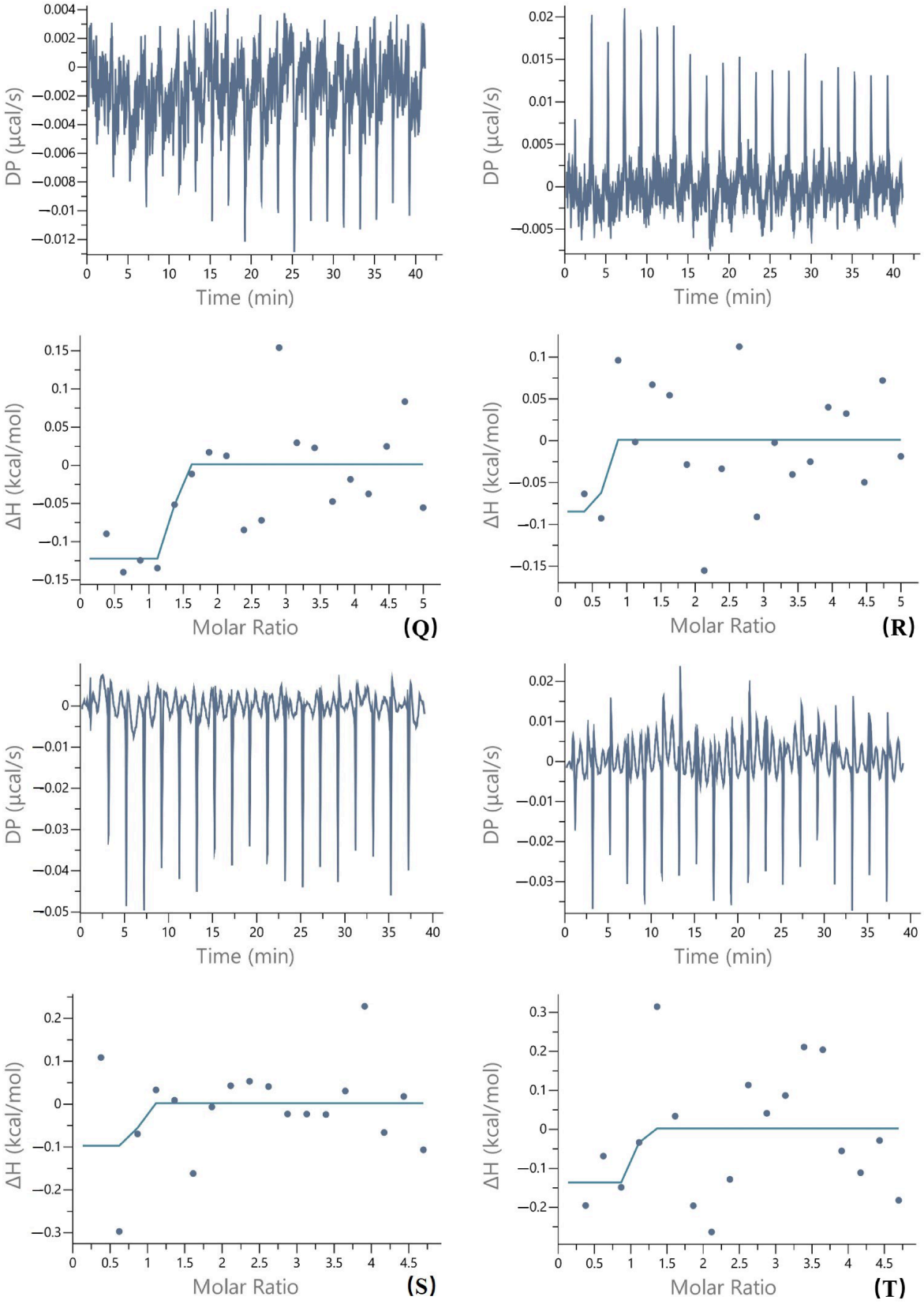

Figure 3. Representative counter-screening thermograms for glyphosate-related compounds against Seq03 and Seq05 under matched ITC settings. Each compound (1 mM in syringe unless noted) was titrated into aptamer solutions (20 µM in cell) using the standard injection protocol in PBS (pH 7.4) at 25 °C. Panels (**A**–**T**) correspond to the compounds listed in Table 1. None of the candidates produced

binding-consistent, saturable behavior after integration and background handling; observed responses remained close to dilution/background contributions (typically $\leq$ 0.06 µcal/s) and were therefore operationally interpreted as no detectable binding under the tested conditions.

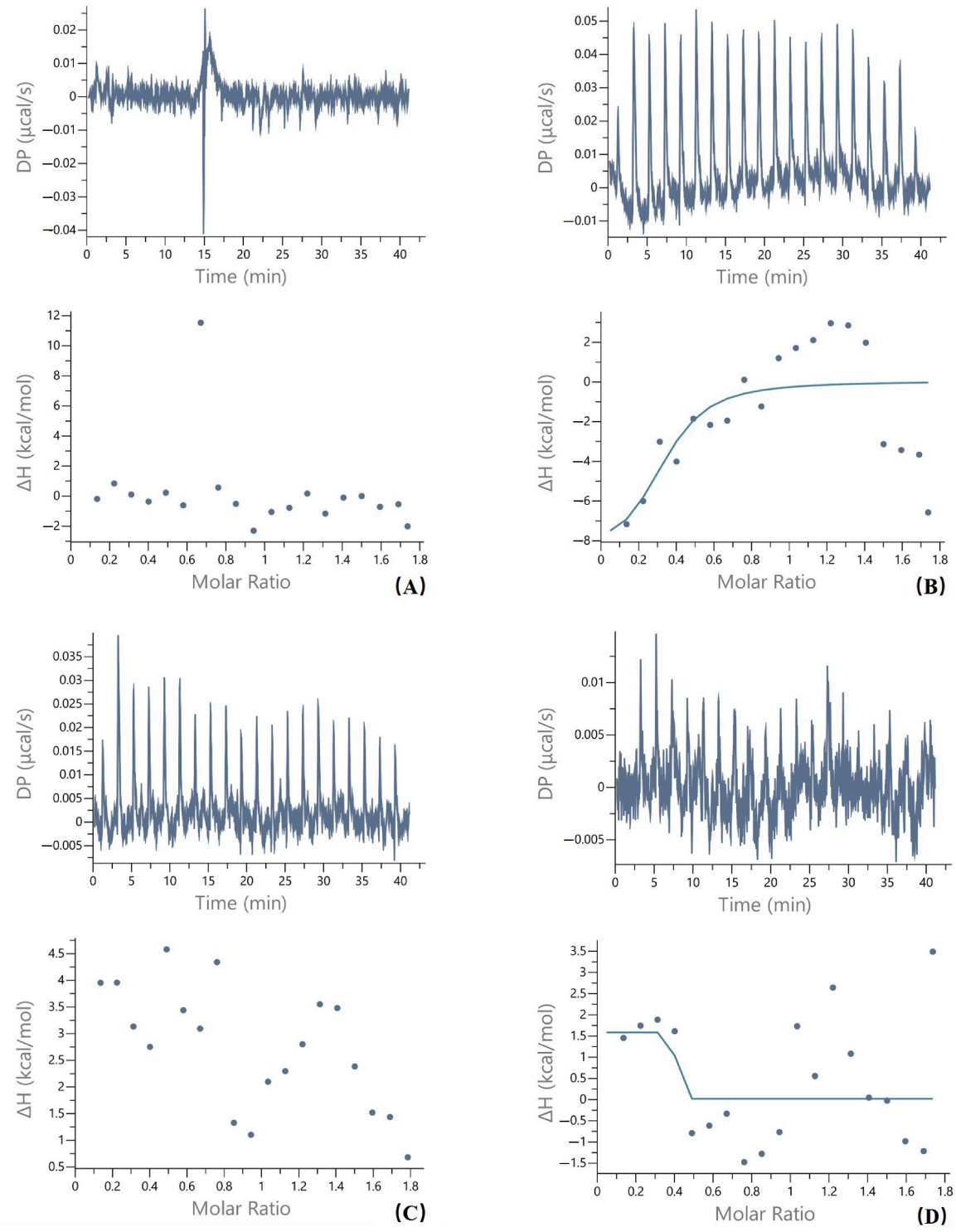

Figure 4. Reverse-titration format check using Glufosinate-N-acetyl within the same ITC platform. Thermograms were recorded by titrating aptamer (100 µM in syringe) into Glufosinate-N-acetyl (5.6 µM in cell) or buffer controls: (**A**) Seq03 into buffer; (**B**) Seq03 into Glufosinate-N-acetyl; (**C**) Seq05 into buffer; and (**D**) Seq05 into Glufosinate-N-acetyl. Only small heat signals were observed (Seq03: ~0.05 µcal/s; Seq05: ~0.01 µcal/s). Although the absolute signal magnitudes differed between Seq03 and Seq05, both responses remained in the near-background regime and did not support a reliable thermodynamic interpretation, consistent with no detectable binding under the tested conditions.

4. Discussion

4.1. Solution-Phase ITC Validation Strengthens Analytical Selectivity Claims

For small-molecule targets, aptamer performance reported in sensing formats does not always reflect intrinsic solution-phase recognition, because immobilization geometry, surface crowding, labeling chemistry, assay-specific signal amplification, and interfacial electrostatics can all influence apparent affinity and selectivity [10,11]. This consideration is particularly relevant for glyphosate, for which reported aptamer candidates have already been advanced into impedimetric, voltammetric, fluorescence/qPCR, and chemiluminescence platforms [14–17]. While these studies demonstrate analytical potential, their outputs remain coupled to specific transduction architectures. In this context, ITC provides a label-free, in-solution readout that can reassess literature-reported candidates under matched conditions and thereby serve as an independent validation layer prior to analytical deployment [18–20].

4.2. Background Handling and Identifiability: Define the Operational Decision Threshold

For highly polar analytes such as glyphosate and related phosphonate/phosphinate species, apparent ITC heats can be influenced by dilution, ionization equilibria, and buffer-dependent linkage effects, making baseline controls and consistent background handling essential for avoiding mis-assignment of small heats as binding [18–20]. In addition, parameter identifiability in ITC depends strongly on experimental design, including concentration relationships and c-value considerations, and can become limited for weaker interactions at practical macromolecule concentrations [21,22]. Methodological guidance and uncertainty frameworks consistently emphasize that when heats remain near background and saturation is not achieved, robust thermodynamic extraction is not warranted; in such cases, results should be interpreted within the tested sensitivity window rather than over-interpreted as weak binding [25–27]. In the present study, this principle directly motivated the operational use of "no detectable binding under the tested conditions" for near-background, non-saturable traces. Automated integration and peak-analysis workflows further support reproducible treatment of thermograms when combined with explicit reporting criteria [23,24].

4.3. Affinity Ranking Under Matched Conditions Supports Candidate Prioritization

Under identical buffer composition and instrument settings, Seq03 yielded a stronger apparent binding-associated response to glyphosate than Seq05, corresponding to apparent K_d values of approximately 8.14 μM and 40.2 μM, respectively. Within the present workflow, this matched-condition ranking is useful for candidate prioritization because it is less sensitive than direct cross-study comparison to differences in buffer composition, injection strategy, signal transduction, and fitting convention [18–22]. At the same time, the present result should be interpreted cautiously: Seq03 and Seq05 differ not only in apparent affinity but also in sequence length and likely folding behavior, so the observed difference should not be treated as an isolated test of length effects. Rather, it provides a standardized benchmark between literature-reported glyphosate aptamer candidates under a common ITC framework. This type of reassessment complements existing glyphosate aptamer platform studies, in which reported analytical performance is often coupled to device architecture and readout strategy [14–17]. Open and reproducible analysis frameworks may further improve cross-study comparability when adopted consistently [28].

4.4. Counter-Screening Restricted to Glyphosate-Related Chemicals Defines a Practical Selectivity Boundary

A major practical challenge in small-molecule aptamer development is avoiding cross-reactivity with structurally related analogues and transformation products, for which limited functional-group diversity can make discrimination intrinsically difficult [10,11]. Rather than screening chemically unrelated compounds, we therefore restricted the counter-screen panel to glyphosate-related chemicals, including structural analogues, metabolites, salts, and derivatives, in order to focus on the highest-risk interferents for real-world glyphosate verification workflows [12,13]. Across this panel, the tested candidates produced near-background heats and did not yield binding-consistent, saturable behavior, supporting an operational conclusion of no detectable binding under the tested conditions.

From a structural perspective, glyphosate combines phosphonate, amine, and carboxyl functionalities in a specific arrangement, whereas related compounds in the present panel each alter or remove part of this motif set. The proposed structural comparison figure added to the revised manuscript is intended to aid interpretation of this distinction, but not to imply that a resolved molecular binding model has been established here. In this sense, the present operational selectivity boundary complements sensor-focused glyphosate aptamer studies by clarifying that intrinsic solution-phase selectivity should be evaluated independently of downstream transduction architecture [14–17].

4.5. Methodological Limitations and Scope of Interpretation

The present ITC workflow is intended as a solution-phase validation layer, and its conclusions should be interpreted within the identifiability limits intrinsic to calorimetric titrations. For interactions that are weak relative to the accessible concentration window, thermograms can approach background contributions (e.g., dilution heats and baseline drift), and non-saturable behavior does not support reliable parameter extraction. In such cases, it is more appropriate to report outcomes as "no detectable binding under the tested conditions" than to fit marginal heats as weak binding. In addition, thermodynamic decompositions such as ΔH and $-T\Delta S$ are model- and protocol-dependent; when heats are small or saturation is incomplete, preprocessing choices and concentration uncertainty can disproportionately influence fitted parameters.

An additional limitation is that PBS was used here as a standardized matched condition for cross-candidate comparison rather than as an individually optimized folding/binding buffer for each aptamer sequence. Because aptamer folding and recognition can be sensitive to ionic strength and to the presence of mono- and divalent cations, alternative buffer conditions, including Mg^{2+} supplementation or ionic-strength adjustment, may influence the apparent thermodynamic response [10,11].

It is also important to position the present results in the broader context of affinity reagents for glyphosate recognition. Antibody-based glyphosate assays and immunosensor formats have been reported for electrochemical and rapid on-site detection, demonstrating that alternative affinity systems can also support selective analytical workflows [29,30]. However, in many such studies, the reported outputs are primarily assay- or device-level analytical signals rather than full solution-phase thermodynamic decompositions directly comparable to ITC-derived K_d, ΔH, or entropy-related terms. For this reason, the present ITC workflow should not be interpreted as replacing antibody-based recognition approaches, but rather as providing a complementary solution-phase validation layer with explicit interpretability boundaries.

Accordingly, the operational conclusions in this study are bounded by the tested buffer composition, instrument settings, and concentration window and should not be extrapolated beyond the present sensitivity scope.

4.6. Reporting-Oriented Recommendations for ITC-Based Aptamer Validation

To improve reproducibility and enable meaningful comparisons across studies, ITC-based validation of small-molecule aptamers should be reported as a workflow with explicit decision rules rather than only as fitted constants. This is especially important when literature-reported aptamer candidates are being reassessed under matched conditions for analytical prioritization. Based on established ITC methodological guidance and recent advances in analysis and uncertainty evaluation, we recommend explicitly reporting the following items [18–28]:

Instrument and run configuration: temperature, reference power, stirring rate, cell/syringe concentrations, injection volumes/spacing, and replicate strategy.

System suitability and baselines: baseline stability checks and the criteria used to accept a run (e.g., stable pre-injection baseline).

Background controls and handling: ligand-to-buffer (heat-of-dilution) and/or aptamer-to-buffer controls, together with a clear statement of how (or whether) background subtraction was applied.

Integration and preprocessing: the software/toolchain used for peak integration and any automated or manual adjustments; if automated tools are applied, their settings and whether reintegration was required are reported [23,24].

Model selection and fit acceptability: binding model(s) evaluated, fit acceptance criteria (e.g., requirement for a binding-consistent and saturable trend; residual inspection), and a statement of identifiability constraints tied to c-value and concentration uncertainty [21,22,25–27].

Operational definitions: a pre-specified definition for "no detectable binding under the tested conditions" that is explicitly linked to the concentration window and the observed background-level heat range, thereby avoiding over-interpretation of near-background heats [18–22,25–27].

Data transparency: where possible, integrated heats and raw thermograms (or sufficient metadata) are provided to enable reanalysis and cross-validation, facilitated by open analysis frameworks for ITC [28].

Together, these reporting elements convert ITC measurements from isolated binding estimates into a reproducible validation workflow suitable for analytical decision-making and candidate prioritization [18–22,25–28].

5. Conclusions

This study does not report the discovery of new glyphosate aptamers; rather, it establishes a standardized, solution-phase ITC workflow for the thermodynamic reassessment of literature-reported glyphosate DNA aptamer candidates. Under matched experimental conditions, both Seq03 and Seq05 produced interpretable calorimetric responses to glyphosate, thereby enabling a practical affinity-based prioritization, within which Seq03 exhibited a stronger apparent binding response and a lower apparent K_d than Seq05 within the tested PBS-based concentration window.

Beyond affinity ranking, the present study further delineates an operational selectivity boundary through a focused counter-screen panel restricted to glyphosate-related chemicals, including structural analogues, metabolites, salts, and derivatives. Because these counter-screen compounds generated only near-background, non-saturable responses, they were operationally classified as exhibiting no detectable binding under the tested conditions, rather than being over-interpreted as weak binders.

From the perspective of analytical validation, the principal contribution of this work lies in the establishment of a reproducible, platform-independent workflow that integrates baseline assessment, background handling, fit interpretability, and explicit reporting criteria

into a coherent framework for benchmarking small-molecule aptamers. Within the broader landscape of glyphosate recognition, this workflow should also be regarded as complementary to antibody-based analytical systems, which remain important in immunoassay and immunosensor applications but typically report assay-level analytical performance rather than directly comparable solution-phase thermodynamic parameters [29,30]. At the same time, the conclusions drawn here are inherently bounded by the tested buffer composition, concentration window, and instrument configuration. Accordingly, the present results are most appropriately interpreted as matched-condition, solution-phase benchmarks for literature-reported glyphosate aptamers, rather than as universally transferable affinity or selectivity constants across disparate assay environments. Overall, ITC-based validation can serve as a valuable pre-translation step for strengthening selectivity claims prior to downstream sensor or assay development.

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