Decoupling Drug Exposure, Binding ADA, and Functional Neutralization in Anti–PD-1 Therapy Using Orthogonal ELISA Immunogenicity Assessment

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ABSTRACT

Background:
Therapeutic monoclonal antibodies targeting the PD-1/PD-L1 axis are associated with immunogenicity risk, resulting in the development of anti-drug antibodies (ADAs) with variable clinical impact. Conventional immunogenicity assessments, including circulating drug quantification and binding ADA detection, do not reliably predict functional impairment of checkpoint blockade. Only a subset of ADAs—neutralizing antibodies (NAbs)—directly interfere with PD-1 interaction and compromise pharmacodynamic activity. Therefore, a mechanistically informative immunogenicity assessment requires orthogonal evaluation of pharmacokinetics (PK), binding immunogenicity, and functional neutralization.

Methods:
A tiered, ICH-aligned ELISA-based immunogenicity framework was implemented using human serum samples spiked with therapeutic antibody. Tier 1 employed a target-capture (PD-1–coated) sandwich ELISA for quantification of total circulating drug (free and target-bound). Tier 2 utilized a bridging immunoassay format for detection of binding ADAs, leveraging bivalent antibody-mediated drug bridging. Tier 3 consisted of a competitive ligand-binding assay (LBA) to quantify neutralizing activity via inhibition of PD-1/PD-L1 interaction. Assays were optimized and qualified for sensitivity, specificity, precision, and drug tolerance in accordance with regulatory expectations.

Results:
Binding ADA responses were detected in multiple samples without corresponding attenuation of PD-1 pathway inhibition, indicating that non-neutralizing ADAs do not necessarily impact pharmacodynamic activity. In contrast, samples positive for NAbs demonstrated a measurable reduction in receptor-ligand blockade, despite the presence of quantifiable circulating drug, consistent with functional inhibition independent of drug exposure. These findings confirm that PK, binding ADA, and NAb responses represent distinct and non-correlative dimensions of immunogenicity.

Conclusion:
This study demonstrates that integrated, tiered immunogenicity assessment is essential for mechanistic interpretation of anti-PD-1 therapies. The combined evaluation of drug exposure, binding ADA incidence, and functional neutralization enables differentiation between altered pharmacokinetics, immune-mediated clearance, and true loss of biological activity. Such an approach aligns with regulatory expectations and provides a robust framework for immunogenicity risk assessment and therapeutic monitoring of immune checkpoint inhibitors.

INTRODUCTION: Immunogenicity Challenge in Anti–PD-1

Immune checkpoint inhibitors targeting programmed death 1 (PD-1) have transformed cancer immunotherapy by restoring T-cell–mediated anti-tumour responses. However, these therapeutic monoclonal antibodies can elicit anti-drug antibodies (ADAs), arising from immune recognition of the drug as a foreign antigen. ADA formation may lead to diverse outcomes, including no effect, altered pharmacokinetics, reduced efficacy, or increased risk of adverse events, making immunogenicity assessment critical in clinical development and therapeutic monitoring.

Conventional immunogenicity strategies - measuring circulating drug levels and detecting binding ADAs - do not reliably predict loss of therapeutic function. Many binding ADAs do not interfere with the drug's mechanism of action. In contrast, neutralizing antibodies (NAbs) represent a functionally relevant subset that directly disrupts PD-1 interaction, compromising checkpoint blockade. Neutralizing antibodies provide a functional readout of interference with PD-1 interaction, serving as a mechanistically relevant indicator of potential loss of therapeutic activity.

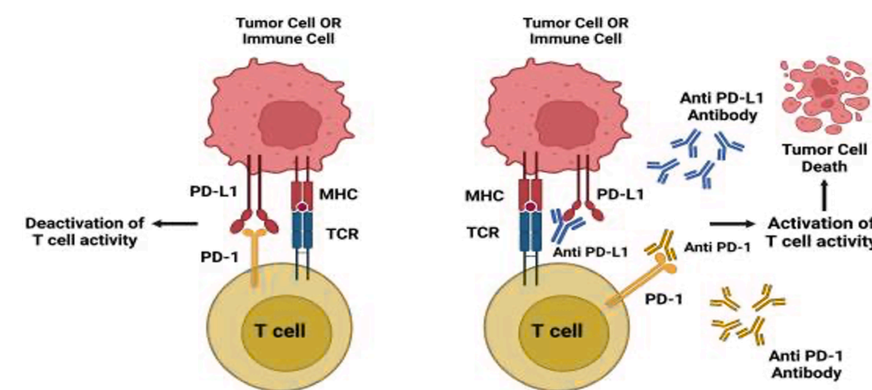


Figure 1: Schematic representation of PD-1/PD-L1 immune checkpoint pathway and its inhibition by therapeutic antibodies.

Therefore, accurate immunogenicity evaluation requires a tiered analytical approach integrating drug exposure (pharmacokinetics), binding ADA detection, and functional neutralization assessment. A tiered approach is essential because no single assay captures all dimensions of immunogenicity, requiring orthogonal measurements to resolve pharmacokinetic, immunological, and functional effects independently. This separation enables mechanistic interpretation of immunogenic responses and their impact on the safety, pharmacology, and efficacy of anti–PD-1 therapies.

This approach provides a framework to interpret pharmacokinetic variability, immunogenicity, and potential functional loss of pharmacodynamic activity.

METHODS AND RESULTS

TIER MECHANISM AND FRAMEWORK

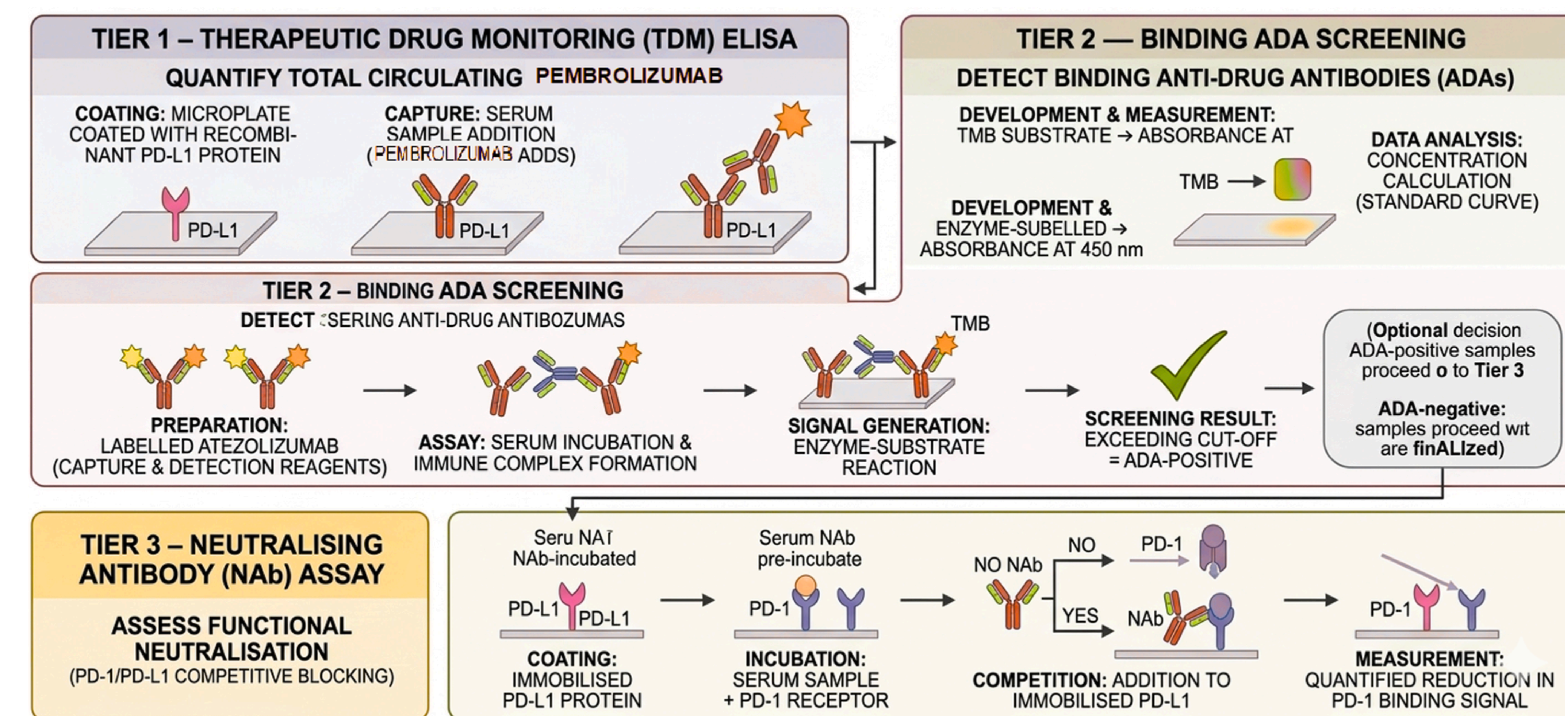


Figure 2: Three Tier Mechanism

An ICH-aligned, tiered ELISA framework was used to evaluate drug exposure, immunogenicity, and functional neutralisation in serum samples containing Pembrolizumab. The workflow comprises three orthogonal assay tiers with non-overlapping readouts to decouple pharmacokinetic, immunological, and functional effects.

Tier 1 – TDM ELISA:
Total circulating drug was quantified using a PD-1 capture sandwich ELISA, with detection via enzyme-labelled anti-human IgG and TMB readout at 450 nm, providing a measure of pharmacokinetic exposure independent of antibody function.

Tier 2 – Binding ADA Assay:
Binding ADAs were detected using a drug-bridging ELISA, where labelled drug molecules form immune complexes in the presence of ADAs, capturing immunogenicity incidence irrespective of functional impact.

Tier 3 – Neutralizing Antibody Assay:
Neutralizing activity was assessed using a competitive ELISA measuring inhibition of PD-1 interaction, providing a functional surrogate of therapeutic mechanism.
Together, these orthogonal assays interrogate distinct biological dimensions—drug presence, immune recognition, and functional interference—enabling interpretation of whether observed effects arise from altered exposure, non-neutralizing ADA binding, or true neutralization of drug activity.

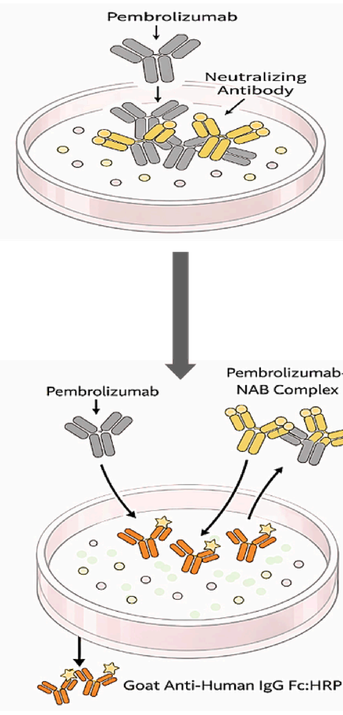


Figure 3: nAbs Assay Principle

CORE EXPERIMENTS

Group A	Negative control serum (no drug, no antibody).
Group B	Drug only (0.5, 2, and 10 ug/ml Pembrolizumab)
Group C	Positive nAb control (anti-Pembrolizumab antibody dilution series).
Group D	Drug + nAb mixtures (to evaluate neutralisation effects).
Group E	Specificity control (irrelevant human IgG).

Experiment 1 – NAb Dose-Response
Samples were analysed using the Neutralizing Anti-Pembrolizumab ELISA to determine the relationship between NAb concentration and assay signal.

Experiment 2 – Residual Functional Activity
Drug + NAb mixtures were tested in the PD-1 blocking ELISA. Increasing NAb levels were expected to reduce pembrolizumab-mediated inhibition of the PD-1 interaction.

Experiment 3 – Drug Tolerance
A fixed NAb concentration (100 ng/mL) was tested with increasing pembrolizumab concentrations to determine the assay's drug tolerance and potential drug interference.

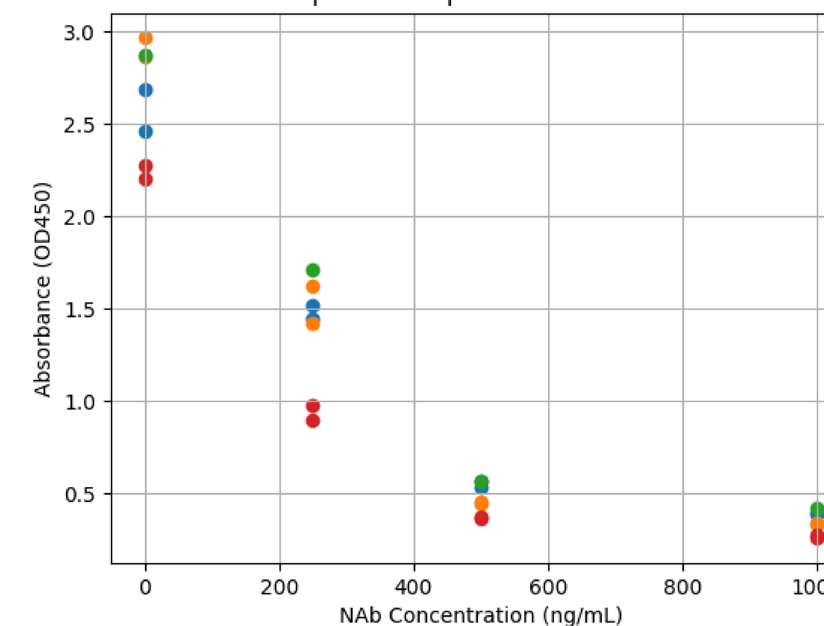


Figure 4: The assay demonstrated strong reproducibility across replicates, with %CV values below 10% across all tested concentrations, indicating robust analytical precision and minimal variability.

NAB ASSAY VALIDATION

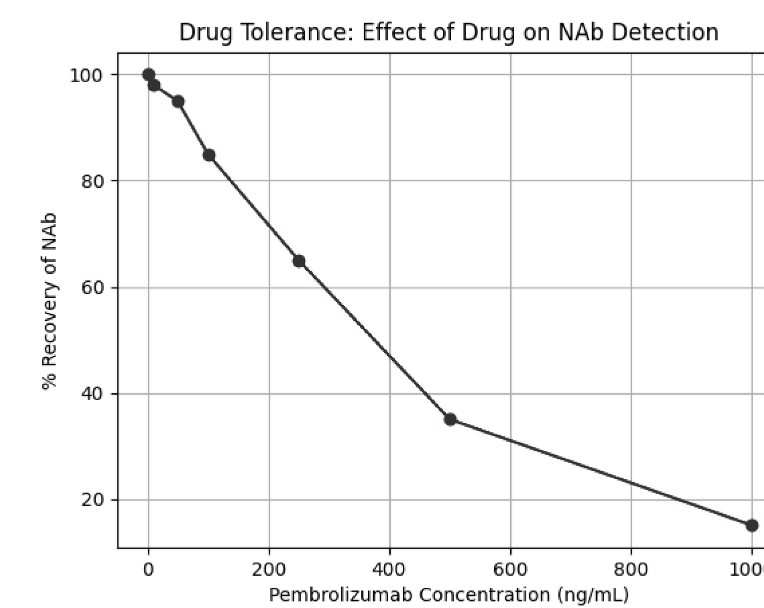


Figure 5: Sample drug-tolerance behavior. At low drug concentrations, recovery of a fixed NAb level remains stable; at higher drug concentrations, excess pembrolizumab is expected to interfere with NAb detection, reducing apparent recovery.

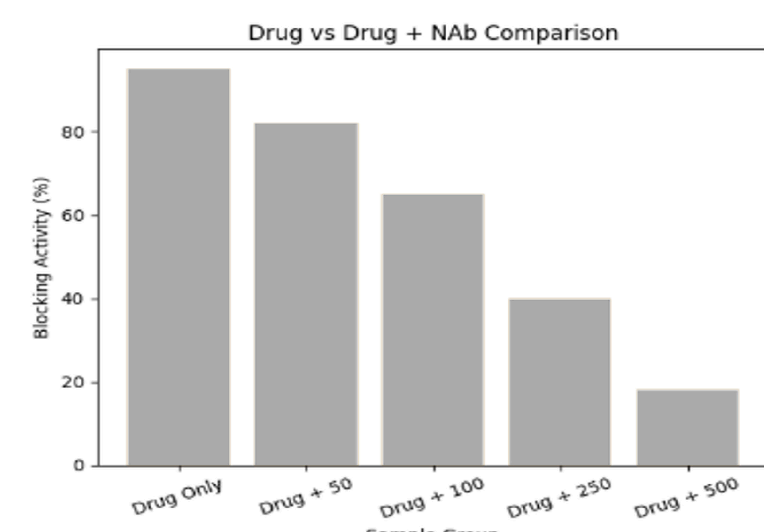
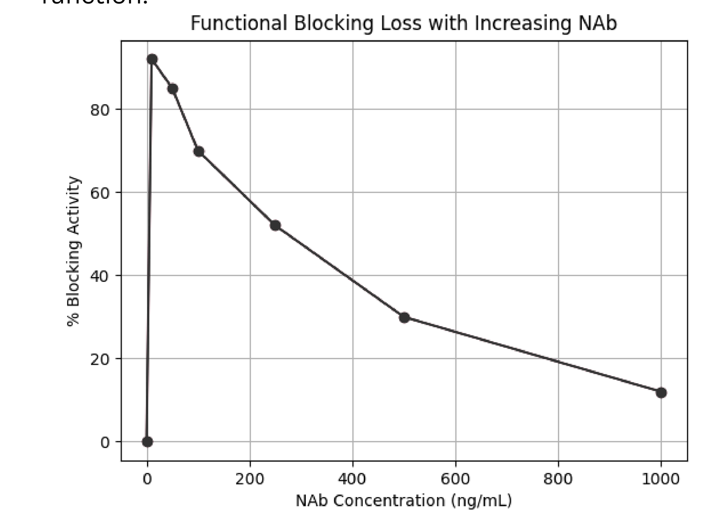


Figure 6: Drug vs Drug + NAb Comparison
bar chart illustrating loss of drug function with increasing NAb levels.

FUNCTIONAL NEUTRALIZATION

Figure 7: Functional analysis demonstrated a corresponding decrease in PD-1/PD-L1 blocking activity, indicating that increasing NAb concentrations directly impair therapeutic function.



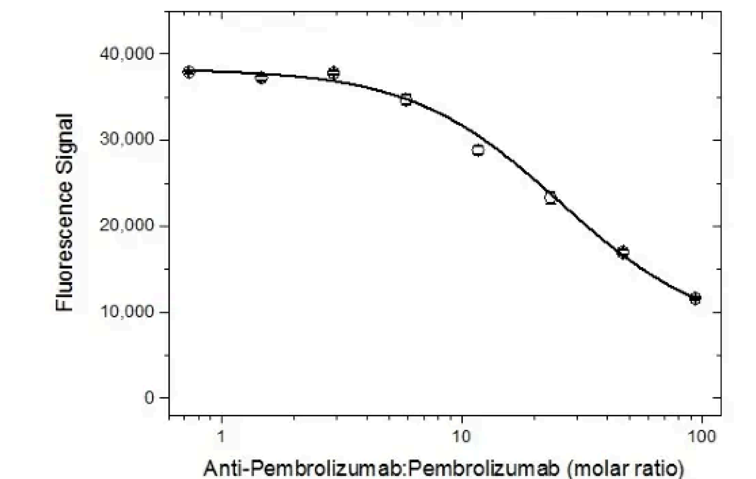
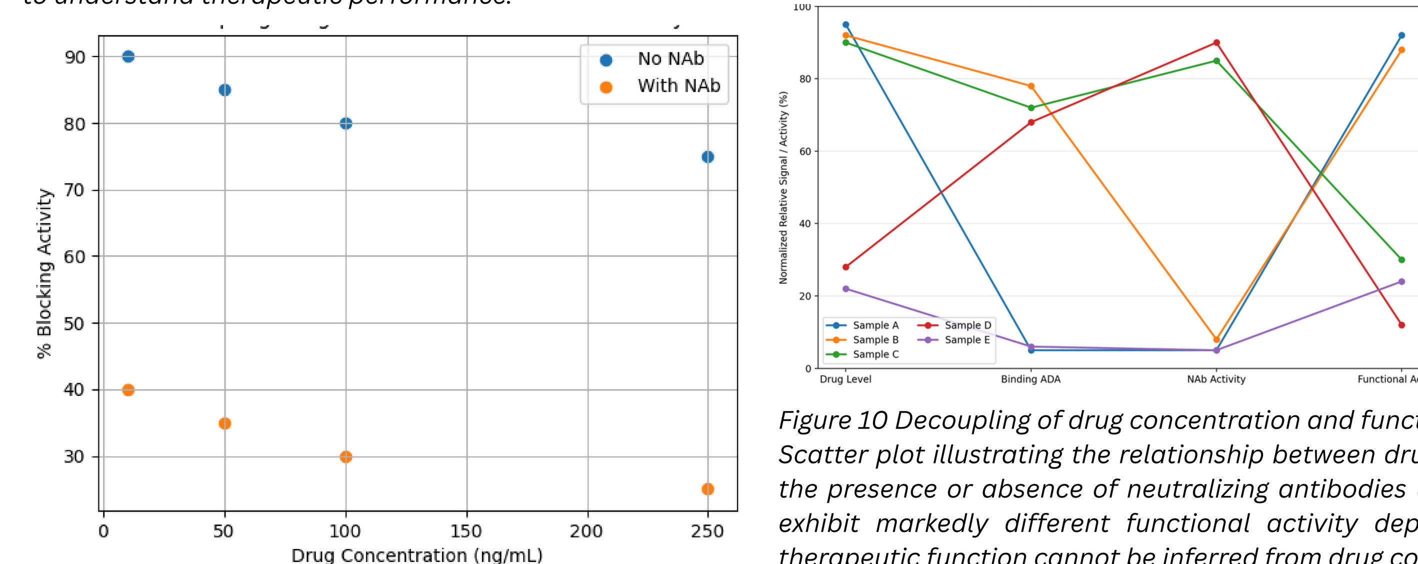


Figure 8: To further confirm functional interference, an inhibition ELISA was performed using human anti-pembrolizumab Fab pre-incubated with pembrolizumab before exposure to immobilized PD-1. Increasing anti-drug antibody:drug molar ratios produced a progressive decline in fluorescence signal, indicating reduced availability of free pembrolizumab for target engagement. This orthogonal inhibition format supports the conclusion that anti-drug antibodies can directly impair therapeutic binding in a concentration-dependent manner.

ORTHOGONAL INTERPRETATION OF IMMUNOGENICITY

Sample	Drug Level (TDM)	Binding ADA	NAb Activity	Functional Activity	Interpretation
Sample A	High	Negative	Negative	Preserved	Drug active, no immunogenicity
Sample B	High	Positive	Negative	Preserved	Binding ADA, no functional impact
Sample C	High	Positive	Positive	Reduced	NAb-mediated neutralization
Sample D	Low	Positive	Positive	Reduced	Clearance + neutralization
Sample E	Low	Negative	Negative	Reduced	Low exposure (PK-driven loss)

Orthogonal assessment reveals that drug exposure, binding ADA, and neutralizing activity are non-redundant parameters that must be interpreted independently to understand therapeutic performance.



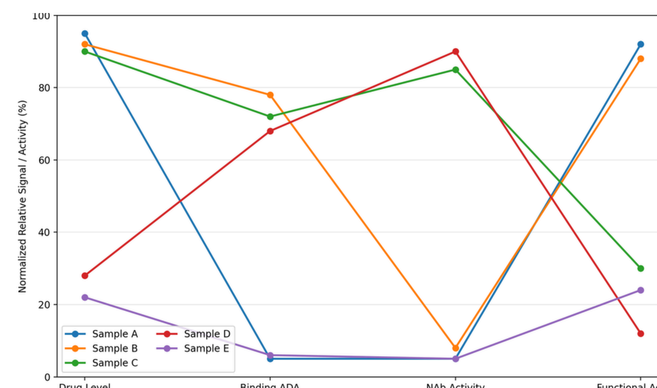


Figure 9
Each parameter is measured differently experimentally, so this plot normalizes them onto a common scale to visualize how interpretation changes across layers.

Figure 10 Decoupling of drug concentration and functional activity.
Scatter plot illustrating the relationship between drug concentration and PD-1 blocking activity in the presence or absence of neutralizing antibodies (NAbs). Samples with comparable drug levels exhibit markedly different functional activity depending on NAb status, demonstrating that therapeutic function cannot be inferred from drug concentration alone.

CONCLUSION

A tiered and functionally informed immunogenicity evaluation is essential for accurate interpretation of responses to anti-PD-L therapy. The combined use of therapeutic drug monitoring, binding ADA assays, and neutralising antibody ELISAs enables clear differentiation between pharmacokinetic effects, immune responses against the therapeutic antibody, and functional disruption of the PD-1 pathway.

This integrated analytical framework strengthens mechanistic insight into treatment outcomes, supports more reliable immunogenicity risk assessment, and enhances the overall evaluation of immune checkpoint inhibitors in both clinical development and therapeutic monitoring.

About Krishgen Biosystems:

Established 2003, Krishgen is an immunoassay manufacturer based out of Mumbai, India. Our key products include assays for mAbs, bispecific antibodies, antibody-drug conjugates, peptides as well as a wide range of cytokine and biomarker ELISA across various species. We also offer customised assay solutions for complex or novel molecules.

Our products are well validated, sensitive, robust and competitively priced. As of April 2025, Krishgen ELISA have been cited in 5000+ publications worldwide. ***Learn more about Krishgen and ELISA we offer at www.krishgen.biz.***