ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH, METABOLIC AND

DIABETES APPLICATIONS

KRISHGEN BioSystems

OUR REAGENTS, YOUR RESEARCH

VALIDATION OF KRIBIOLISA® INSULIN ASPART ELISA KIT (CATALOG NO. KBI2002) AS PER FDA/ICH GUIDELINES FOR BIOANALYTICAL METHOD VALIDATION

This validation protocol has been adopted in line with the Methodology and Analytical Procedures Guideline recommended by FDA/ICH.

Document History

First Codification	History	Date
Version#1	**VALIDATION DATA OF KRIBIOLISA® INSULIN ASPART ELISA (Cat No # KBI2002)**	20.03.2026

Approved Quality Control	Approved Product Development	Approved Operations Head
Prairna B	Atul G	K Jain

Introduction

This document presents a discussion of the characteristics of our **KRIBIOLISA® Insulin Aspart ELISA (Catalog No KBI2002)** kit considered by us during the validation of this kit in accordance with ICH Q2 (R1) guidelines. The document is prepared based on tests run in our laboratory and does not necessarily seek to cover the testing that may be required at user's end for registration in, or regulatory submissions. The objective of this validation is to demonstrate that it is suitable for its intended purpose - detection of **Insulin Aspart.**

Validation characteristics considered by us in accordance with the guidelines are listed below:

- **Assay Validation**
- **Standard Curve**
- **Pharmacokinetic Relevance**
- **Precision and Reproducibility**
- **Cross Reactivity**

The degree of revalidation required depends on the nature of the changes. Certain other changes may require validation as well.

Please note that this validation is performed in our laboratory and will not necessarily be duplicated in your laboratory. This data has been generated to enable the user to get a preview of the assay and the characteristics of the kit and is generic in nature. We recommend that the user performs at the minimum; the spike and recovery assay to assure quality results. For a more comprehensive validation, the user may run the protocols as suggested by us herein below to develop the parameters for quality control to be used with the kit.

For any queries or support on the data and its performance, please contact us at sales1@krishgen.com

Background

Insulin aspart is produced by recombinant DNA technology. It is a rapid-acting analogue of human insulin created by substituting the proline residue at position B28 of the B-chain with aspartic acid. This single amino-acid modification reduces the tendency of insulin molecules to self-associate into hexamers, allowing the insulin to remain predominantly in monomeric form after subcutaneous injection. As a result, insulin aspart is rapidly absorbed into the bloodstream, leading to a fast onset of action, an early peak effect, and a short duration of action. These pharmacokinetic properties make insulin aspart particularly suitable for controlling postprandial blood glucose levels.

1. Purpose

To assess the specificity, assay performance, and clinical relevance of the KRIBIOLISA® Insulin Aspart ELISA developed using Recombinant Human Canine/Porcine Insulin antibody as capture antibody.

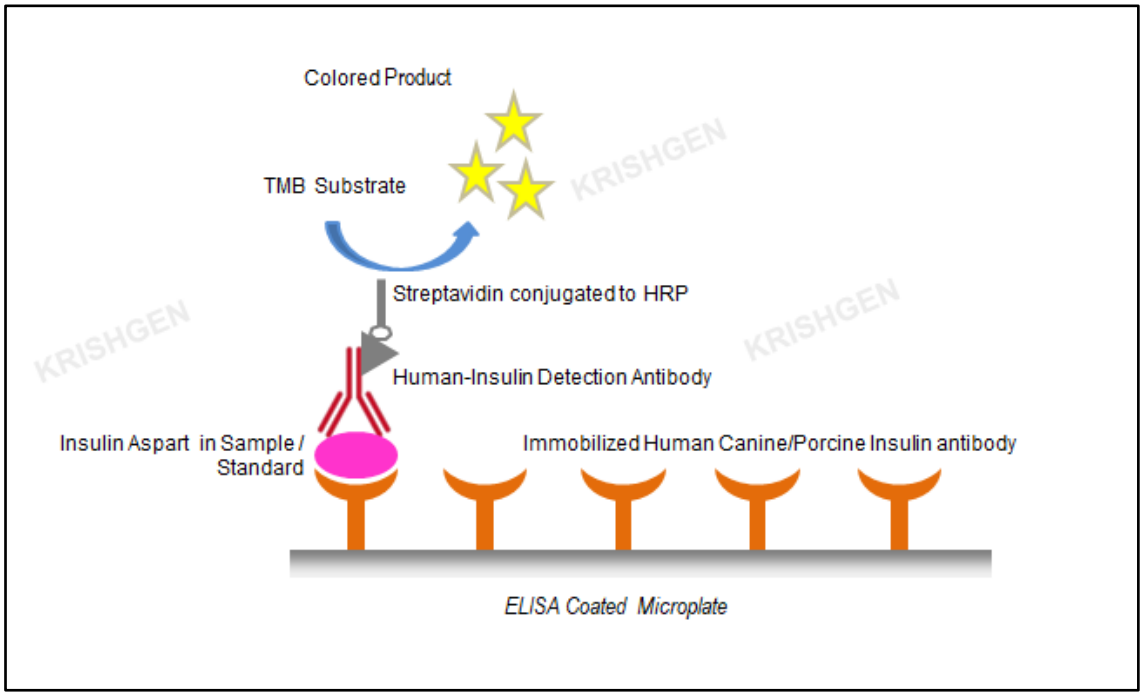
2. Experimental Design

- `A sandwich ELISA was performed using Recombinant Human Canine/Porcine Insulin antibody as capture antibody.

- Standards prepared for Insulin Aspart.
- Assay Concentration Range: 0 - 1000 pg/ml.
- Signal (% absorbance) plotted versus concentration.

The KRIBIOLISA Insulin Aspart ELISA employs a targeted immobilisation strategy to ensure optimal presentation of insulin aspart on the assay plate, thereby enhancing the selective detection of anti–insulin aspart antibodies (or insulin aspart itself, depending on assay format). The immobilisation procedure is carefully optimised to preserve the native-like conformation, structural integrity, and epitope accessibility of insulin aspart, a rapid-acting insulin analogue differing from human insulin by a single amino acid substitution (ProB28 → Asp). This controlled presentation maintains the functional orientation of the molecule, ensuring that clinically relevant epitopes are accessible for high-affinity binding interactions.

Under the optimised assay conditions, insulin aspart participates in highly specific antigen–antibody interactions, forming stable complexes with antibodies that recognise its unique structural and conformational determinants. In contrast, antibodies directed against other insulin analogues or human insulin that do not recognise the specific epitopes or conformational features of insulin aspart may exhibit reduced or negligible binding in this plate-bound format. This differential binding behaviour reflects the precise epitope specificity associated with insulin aspart, as well as the controlled conformation and orientation achieved during the immobilisation process. Collectively, this ensures high assay selectivity, minimal cross-reactivity, and robust analytical specificity for insulin aspart detection.



ELISA kits for Insulin Aspart estimation offered by KRISHGEN uses Human Canine/Porcine Insulin antibody capture as above

3. Assay Validation

- IC50 Value: ~ 561 pg/ml (within 0-1000 pg/mL assay range).
- LLOQ: ~ 11.21 pg/ml.

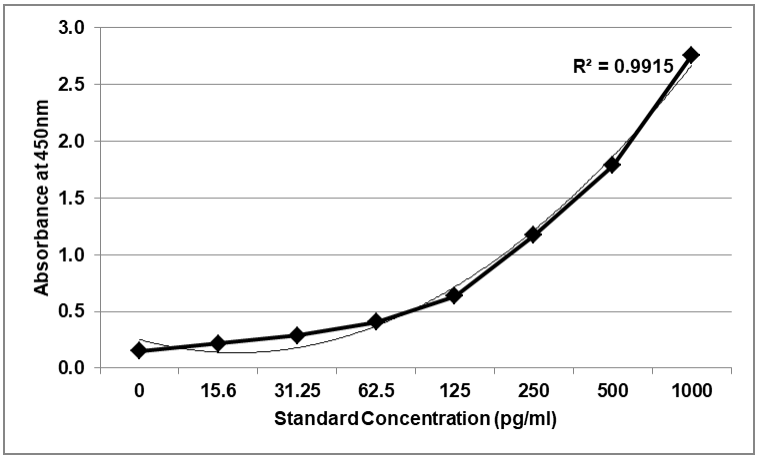
- Clinical Cmax Values*:
- After a single subcutaneous dose (rapid-acting prandial use; e.g., 0.1–0.2 U/kg): ~40–80 µU/mL (≈0.25–0.5 ng/mL), typically achieved within ~40–50 minutes post-injection
- After repeated subcutaneous dosing in a basal–bolus regimen (pre-meal administration): steady-state Cmax ~50–100 µU/mL (≈0.3–0.6 ng/mL), depending on dose and meal timing
- After continuous subcutaneous insulin infusion (CSII; insulin pump therapy): peak levels are less pronounced, with fluctuating plasma concentrations generally ranging ~30–90 µU/mL (≈0.2–0.55 ng/mL)
* Values are approximate and may vary depending on dose, injection site, timing relative to meals, metabolic status, insulin sensitivity, and inter-individual variability.
** published data*

- Precision:
  - Intra-Assay CV: <3.8%.
  - Inter-Assay CV: <7.8%.
  - Inter-Operator CV: <10%.

4. Standard Curve

Below is the standard curve for Insulin Aspart Sandwich ELISA assay:
Linearity and Range

Standard Concentration (pg/ml)	Mean Absorbance	Interpolated Concentration	%Interpolated Concentration against Actual Concentration
0	0.152	1.3	--
15.62	0.216	16.1	103.2
31.25	0.286	32.2	103.1
62.5	0.404	59.9	95.8
125	0.636	117.0	93.6
250	1.169	266.9	106.8
500	1.789	487.3	97.5
1000	2.762	1004.5	100.4



5. LOD and LOQ

- LOD Absorbance: (Approx ~3.70 pg/ml)
- LOQ Absorbance: (Approx ~11.21 pg/ml)

6. Pharmacokinetic Relevance

The assay is designed to cover the clinically relevant serum concentrations of Insulin Aspart observed following subcutaneous therapeutic administration, making it suitable for pharmacokinetic evaluation and therapeutic monitoring.

The Insulin Aspart ELISA demonstrates high sensitivity in the ng/mL range, which aligns with the expected circulating levels of rapid-acting insulin analogs, ensuring accurate quantification across clinically meaningful exposure levels.

Published pharmacokinetic data for Insulin Aspart indicate systemic exposure consistent with rapid absorption and short duration of action:

- Following subcutaneous administration, peak serum concentrations (Cmax) are typically reached within ~1–3 hours, depending on dose and patient physiology.
- Reported Cmax values generally fall within the range of approximately 50–300 ng/mL, depending on dose, injection site, and metabolic status.
- With repeated dosing (e.g., mealtime administration), transient peaks are observed rather than true steady-state accumulation due to the short half-life.
- Exposure levels may vary based on dose, meal timing, injection site, insulin sensitivity, and inter-individual variability.

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7. Precision and Reproducibility

Precision was assessed by analysing three standard concentrations (15.6 pg/ml, 125 pg/ml, and 1000 pg/ml). Each concentration was tested in triplicate across three independent assay runs. %CV (Coefficient of Variation) was calculated within runs (intra-assay precision) and across runs (inter-assay precision).

Acceptance Criteria:

- Intra-assay %CV should be ≤15% for samples.
- Inter-assay %CV should be ≤15% for samples.
- %CV at LLOQ (Lower Limit of Quantitation) allowed up to 20%.

Precision Results Summary:

Standard (pg/ml)	Intra-Assay %CV (Range)	Inter-Assay %CV
15.6	<12%	<12%
125	<10%	<10%
1000	<10%	<10%

Observations:

- Intra-assay precision was consistently less than 12% across all levels tested.
- Inter-assay precision was consistently less than 10%.
- All precision values met the acceptance criteria for ELISA validation.

Conclusion:
The KRIBIOLISA® Insulin Aspart ELISA demonstrates excellent intra- and inter-assay precision. These results support the assay's reliability and reproducibility for routine use in pharmacokinetic and bioanalytical studies.

8. Cross-Reactivity:

This study evaluated insulin ELISA performance relative to endogenous insulin baseline using a matrix-matched zero calibrator (1:1000 human serum). Spike recovery using insulin aspart was assessed in normal and diabetic serum. Normal serum recovery ranged 92–117%, while diabetic serum showed elevated recovery (113–159%), indicating matrix-enhanced signal. The assay accurately quantifies insulin relative to endogenous baseline, but matrix effects persist in diabetic samples.

Sandwich ELISA was used. Calibration employed insulin aspart. The zero calibrator (0 pg/mL) consisted of 1:1000 diluted human serum without added insulin and was treated as endogenous baseline. Spike recovery was performed at 15.6, 62.5, 500, and 1000 pg/ml.

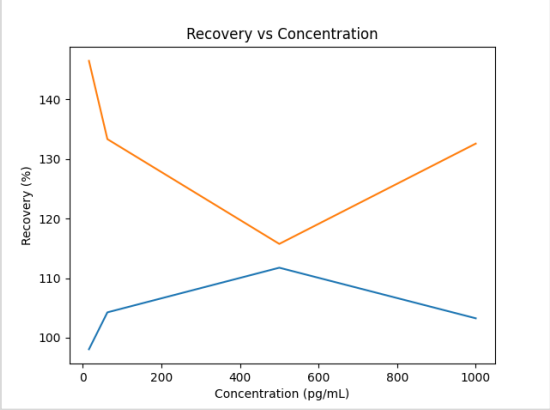
Results:

Table 1. Raw Recovery Data (%)

Spike (pg/ml)	Normal_Exp1	Normal_Exp2	Diabetic_Exp1	Diabetic_Exp2	Normal_Mean	Diabetic_Mean
15.6	92.3	103.8	134.0	159.0	98.05	146.5
62.5	105.3	103.2	129.3	137.4	104.25	133.35
500.0	106.7	116.8	113.1	118.4	111.75	115.75
1000.0	107.7	98.8	131.6	133.6	103.25	132.6

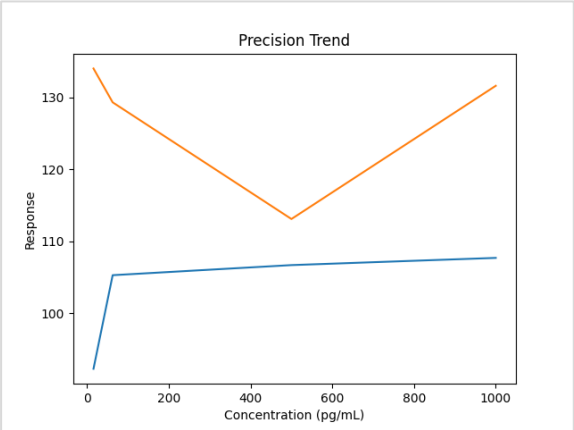
Recovery data relative to endogenous baseline in normal and diabetic serum.

Figure 1. Recovery vs Concentration



Recovery of insulin aspart relative to endogenous baseline. Normal serum shows acceptable recovery, while diabetic serum demonstrates matrix-enhanced response.

Figure 2. Precision Trend

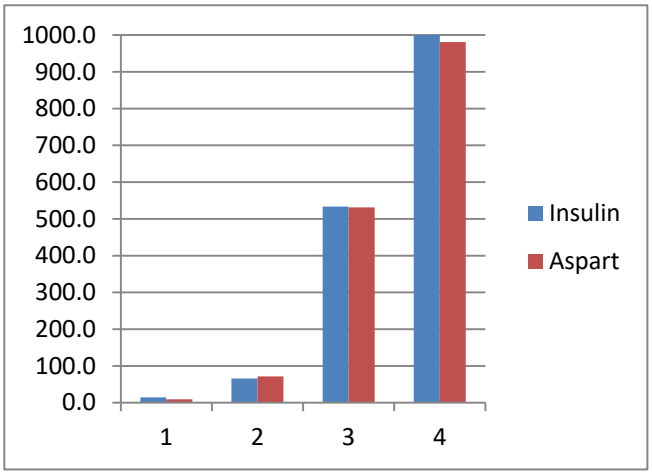


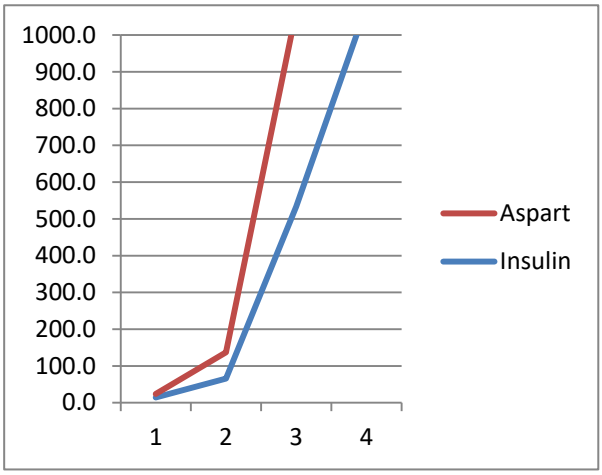
Precision trend across concentrations showing increased variability in diabetic serum.

Table 2. Recovery (%) of Insulin Aspart against Exogenous (recombinant) Insulin

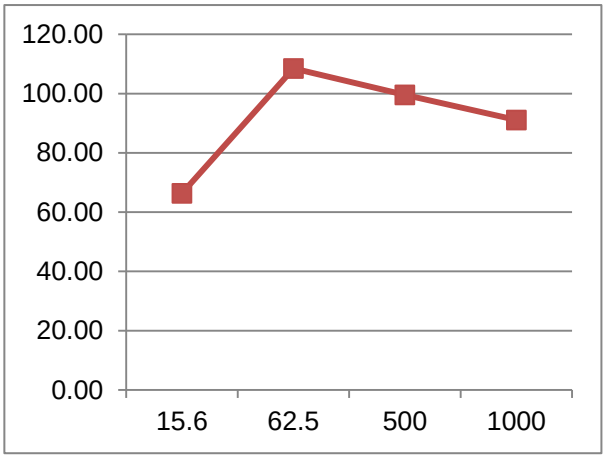
A*		B*		
Abs	Recombinant Human Insulin Recovered Conc (pg/ml)	Abs	Recombinant Aspart Recovered Conc (pg/ml)	% Recovery of Aspart against Recombinant Insulin in normal human serum
0.312	14.4	0.275	9.5	66.33
0.625	65.8	0.654	71.4	108.50
2.002	533.6	1.998	531.4	99.58
2.698	1076.9	2.605	981.1	91.10

A* = Normal Human serum with 1:1000 dilution and spiked with recombinant human Insulin
B* = Normal Human serum with 1:1000 dilution and spiked with recombinant human Aspart





Concentration Spiked for Recombinant Insulin / Aspart)pg/ml)	% Recovery of Aspart against Recombinant Insulin in normal human serum
15.6	66.33
62.5	108.50
500	99.58
1000	91.10



The recovery of insulin aspart was evaluated relative to recombinant human insulin in normal human serum (1:1000 dilution). Across the analytical range (62.5–1000 pg/ml), insulin aspart demonstrated comparable recovery to human insulin, with values ranging from 91% to 108% (mean ~100%). At the lowest concentration (15.6 pg/ml), reduced recovery (~66%) was observed, consistent with assay variability near the lower limit of quantification.

These results indicate that the ELISA exhibits equivalent detection of insulin aspart and human insulin within the validated working range.

Discussion:

The assay accurately measures insulin relative to endogenous baseline in normal serum. Elevated recovery in diabetic serum indicates residual matrix effects beyond baseline normalization, likely due to protein interactions.

Conclusion:

The ELISA is suitable for insulin quantification relative to endogenous baseline. Matrix effects in diabetic serum should be considered. The Assay also effectively measures recombinant Insulin and Insulin Aspart independently in the sample.

8. Conclusion

The KRIBIOLISA® Insulin Aspart ELISA is validated for sensitivity, specificity, precision, and accuracy, and is appropriate for pharmacokinetic applications in regulatory settings.

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Version 1.0, dated 20.03.2026