ELISA VALIDATION GUIDE

ASSAY FOR USE IN

DRUG DISCOVERY RESEARCH, BIOPHARMA APPLICATIONS

KRISHGEN *BioSystems*

OUR REAGENTS, YOUR RESEARCH

VALIDATION OF KRIBIOLISA® ANTI-OBINUTUZUMAB (GAZYVA™) ELISA KIT (CATALOG NO. KBI2058) 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® ANTI-OBINUTUZUMAB (GAZYVA™) ELISA (Cat No # KBI2058)**	30.06.2024

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® Anti-Obinutuzumab (GAZYVA™) ELISA (Catalog No KBI2058)** 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 **Anti-Obinutuzumab.**

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

- ○ **Assay Validation**
- ○ **Standard Curve**
- ○ **Pharmacokinetic Relevance**
- ○ **Precision and Reproducibility**

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

Anti-Obinutuzumab antibodies are anti-drug antibodies (ADAs) that may develop in a small proportion of patients receiving obinutuzumab, a humanized IgG1 monoclonal antibody targeting CD20 and marketed as Gazyva. Obinutuzumab is approved for the treatment of chronic lymphocytic leukemia (CLL) and certain types of non-Hodgkin lymphoma, and is administered by intravenous infusion according to the recommended dosing schedule. Clinical studies have shown that the incidence of treatment-emergent anti-obinutuzumab antibodies is low, and when detected, they are typically present at low titers with no consistent or clinically meaningful impact on pharmacokinetics, safety, or therapeutic efficacy.

1. Purpose
To assess the specificity, assay performance, and clinical relevance of the KRIBIOLISA® Anti-Obinutuzumab (GAZYVA™) ELISA developed using Obinutuzumab as capture protein.

2. Experimental Design
- ` A sandwich ELISA was performed using obinutuzumab as the capture protein..

- Standards prepared using anti-obinutuzumab reference antibodies.
- Assay Concentration Range: 0–640 ng/mL.
- Signal (% absorbance) plotted versus concentration.

The KRIBIOLISA Anti-Obinutuzumab ELISA employs a targeted immobilization strategy to ensure optimal presentation of obinutuzumab on the assay plate, thereby enhancing the selective binding of anti-obinutuzumab antibodies. The immobilization procedure is designed to preserve the native conformation and epitope accessibility of obinutuzumab, maintaining its structural integrity and functional orientation. This controlled presentation ensures that obinutuzumab is displayed in a configuration that supports high-affinity interaction with anti-obinutuzumab binding domains.

Anti-obinutuzumab antibodies demonstrate strong and stable antigen–antibody complex formation under these plate-bound conditions. In contrast, antibodies directed against unrelated therapeutic monoclonal antibodies or non-specific human IgG molecules may exhibit reduced or negligible binding. This differential binding behavior reflects the high specificity of anti-obinutuzumab antibodies for obinutuzumab and the preserved structural orientation of the immobilized molecule established during the coating process.
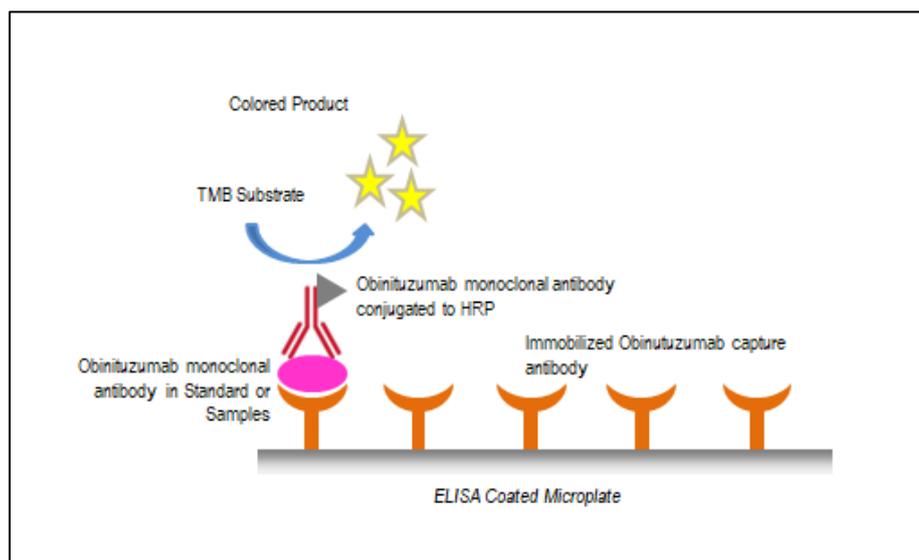
ELISA kits for Anti – Ominutuzumab estimation offered by KRISHGEN uses Obinutuzumab capture proteins as above

3. Assay Validation
- IC50 Value: ~ 36.8 ng/ml (within 0-500 ng/mL assay range).
- LLOQ: ~ 7.80ng/ml.

- Clinical Cmax Values*:
   - After standard obinutuzumab dosing (e.g., 1000 mg per cycle for chronic lymphocytic leukemia or lymphoma): anti-obinutuzumab antibodies are typically low or undetectable.
   - After alternative or weight-based dosing regimens: detected anti-obinutuzumab antibody levels remain low and are not dose-dependent.
   - At steady state (following repeated cycles of therapy): no clinically meaningful accumulation of anti-obinutuzumab antibodies has been observed.

* Values are approximate and may vary depending on dosing regimen, treatment duration, and individual patient variability.
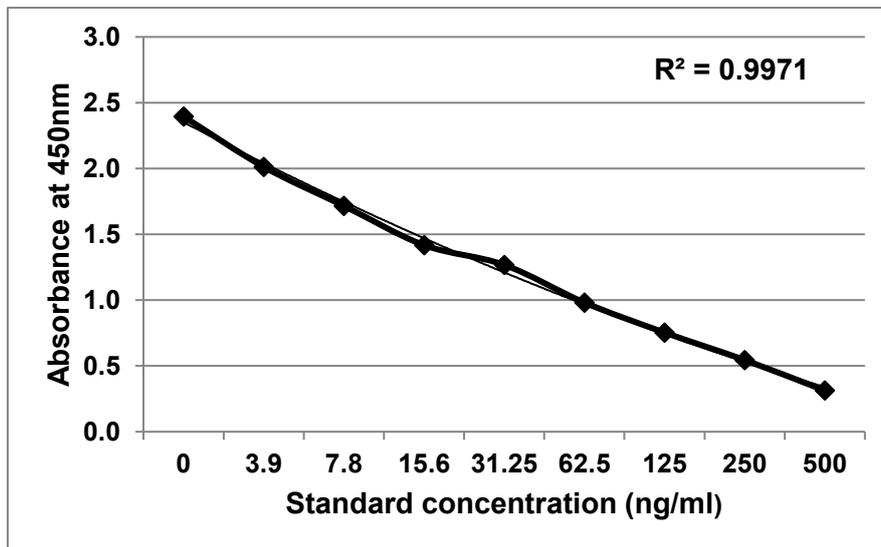 * *published data*

- Precision:
  - Intra-Assay CV: <6.80%.
  - Inter-Assay CV: <12.00%.
  - Inter-Operator CV: <10%.

4. Standard Curve

Below is the standard curve for Anti – Obinutuzumab Sandwich ELISA assay:
Linearity and Range

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	2.395	--	--
3.9	2.013	2.7	69.8
7.8	1.716	8.4	108.0
15.6	1.418	19.9	127.3
31.25	1.268	29.4	94.2
62.5	0.982	61.8	98.8
125	0.754	116.0	92.8
250	0.545	225.6	90.3
500	0.313	596.0	119.2

5. LOD and LOQ
- LOD Absorbance: (Approx ~3.90 ng/ml)
- LOQ Absorbance: (Approx ~7.80 ng/ml)

6. Pharmacokinetic Relevance
The assay is designed to detect clinically relevant anti-obinutuzumab antibody concentrations observed in patients receiving obinutuzumab therapy, making it suitable for immunogenicity assessment and therapeutic monitoring. The Anti-Obinutuzumab ELISA demonstrates high sensitivity within the ng/mL range, which falls well within the validated assay range, ensuring accurate detection and quantification of low-titer anti-drug antibodies (ADAs). Published clinical immunogenicity data for obinutuzumab indicate that treatment-emergent anti-obinutuzumab antibodies are rare and generally present at low concentrations:
• Following standard intravenous dosing (e.g., 1000 mg per cycle), detected antibody levels are typically low or undetectable.
• With repeated dosing at steady state, no clinically meaningful accumulation of anti-obinutuzumab antibodies has been observed.
• Variability may occur depending on dosing regimen, treatment duration, and individual patient factors.
Thus:
• At clinically relevant dosing regimens, anti-obinutuzumab antibody levels fall within the measurable range of the ELISA, without the need for extensive sample dilution.
• The assay working range enables reliable differentiation between negative and positive samples, including low-titer responses.
• Given the generally low circulating levels of anti-obinutuzumab antibodies, routine sample handling is sufficient to maintain measurements within the linear dynamic range of the assay.
• The assay is therefore suitable for immunogenicity evaluation, ADA monitoring, and supportive clinical assessment of obinutuzumab therapy in human serum or plasma.

7. Precision and Reproducibility
Precision was assessed by analysing three standard concentrations (3.9 ng/ml, 31.25 ng/ml, and 500 ng/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 (ng/ml)	Intra-Assay %CV (Range)	Inter-Assay %CV
3.9	0.3 % to 2.2%	<2.2%
31.25	1.1% to 3.0%	<3%
500	0.5% to 12.0%	<12%

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

Conclusion:
The KRIBIOLISA® Anti-Obinutuzumab (GAZYVA™) 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. Conclusion

The KRIBIOLISA® Anti-Obinutuzumab (GAZYVA™) 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 30.06.2024