KRISHGEN BioSystems

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VALIDATION OF KRIBIOLISA™ BEVACIZUMAB ELISA KIT AS PER ICH Q2 (R1) METHODOLOGY & ANALYTICAL PROCEDURES GUIDELINES

This validation protocol has been adopted in line with the Methodology and Analytical Procedures Guideline Q2(R1) developed by ICH Expert Working Group.

Document History

First Codification	History	Date
Version#1	**VALIDATION DATA OF KRIBIOLISA™ Bevacizumab ELISA (Cat No # KBI1016)**	14st 01, 2016
Version#2	**Approved by QC and renamed as per ICH guidelines Q2(R1)**	27th Mar, 2016
Version#3	**Approved by QC and renamed as per ICH guidelines Q2(R1)**	14th Mar, 2018

Approved Quality Control	Approved Product Development	Approved Operations Head
14.01.2016	27.03.2016	14.03.2018

Introduction

This document presents a discussion of the characteristics of our KRIBIOLISA™ BEVACIZUMAB ELISA 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 Bevacizumab.

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

- **Sensitivity**
- **Specificity / Cross reactivity**
- **Precision**
- **Recovery**
- **Traceability / Stability**
- **Hook Effect**

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 sales@krishgen.com

1. Sensitivity:

The Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantitation (LoQ) were determined as per standard protocol of Laboratories of KRISHGEN BIOSYSTEMS.

To estimate LoB, five measure and -free human serum samples were measured in six runs with three different reagent lots (n = 60 replicates per reagent lot). LoB was calculated as the highest 95th percentile value from the three reagent lots. The LoB was determined to be 0.3 ng/ml.

To determine LoD, five human serum samples with low analyte values were measured with three different reagent lots (n = 60 replicates per reagent lot). LoD was calculated from the following equation: LoD = LoB + 0.093 x (total standard deviation). The LoD was determined to be 1.0 ng/ml.

The Limit of Quantitation (LoQ) was determined using six pooled human serum samples that were diluted to have Bevacizumab concentrations between 1.56-100 ng/ml. Each sample

was measured in six runs, with three different reagent lots (n = 72 replicates per reagent lot). The detection limit values are presented in the table below:

LoB	LoD	LoQ
0.3 ng/ml	1.0 ng/ml	1.56 ng/ml

2. Specificity / Cross Reactivity:

Specificity of an analytical method is defined as its ability to measure an analyte accurately in the presence of interference.

The kit uses Human Anti-Bevacizumab Antibodies which are paratope specific, recombinant, anti-idiotypic antibody that specifically recognize the monoclonal antibody drug bevacizumab.

3. Precision:

3.1 Precision/Reproducibility:

Total Imprecision: Six human sera were measured with two replicates and two runs per day for 20 days (n = 80). The human sera were pooled patient and single donor spiked samples. Samples were measured using one lot of reagent, in three laboratories of KRISHGEN BIOSYSTEMS. All data met our acceptance criteria for % CV and 95% confidence intervals for % CV.

Sample	Mean (ng/mL)	Within-Run		Between-Run		Between-Day		Total	
		SD	CV	SD	CV	SD	CV	SD	CV
1	3.1	0.09	2.8%	0.05	1.5%	0.08	2.7%	0.13	4.2%
2	14.7	0.43	2.9%	0.15	1.0%	0.38	2.6%	0.59	4.0%
3	35.0	0.69	2.0%	0.27	0.8%	0.72	2.1%	1.04	3.0%
4	99	0.52	2.5%	8.24	0.3%	52.3	2.2%	79.6	3.3%
5*	61	0.13	0.9%	0.79	0.7%	0.63	0.5%	1.52	1.3%
6*	72	0.61	1.1%	1.88	0.6%	1.80	0.5%	4.45	1.3%
**Samples were tested at a site that is different from the first four samples*									

3.2 Site-to-Site Reproducibilty:

Five human sera were measured with two replicates and two runs per day for 10 days (n= 40), using one lot of reagent and in three laboratories of KRISHGEN BIOSYSTEMS. The human sera were pooled patient and single donor spiked samples. At Laboratory 1, the total precision for the five samples ranged from 1.4%–1.7% CV. At Laboratory 2, the total precision for the five samples ranged from 0.9%–1.5% CV. At Laboratory 3, the total precision for the five samples ranged from 2.4%–3.0% CV. The combined site-to-site reproducibility parameters are detailed in the table below. All values met the manufacturer's acceptance criteria.

Sample	Mean (ng/mL)	Within-Run		Between-Run		Between-Day		Between-Sites		Total	
		SD	CV	SD	CV	SD	CV	SD	CV	SD	CV
1	15.3	0.17	1.1%	0.13	0.8%	0.24	1.6%	0.40	2.6%	0.52	3.4%
2	48.6	0.49	1.0%	0.36	0.7%	0.64	1.3%	1.29	2.6%	1.56	3.2%
3	49.2	0.39	0.8%	0.39	0.8%	0.75	1.5%	1.39	2.8%	1.68	3.4%
4	72	1.04	0.9%	0.89	0.7%	1.57	1.3%	2.45	2.0%	3.22	2.6%
5	99	3.14	1.0%	2.01	0.6%	4.41	1.3%	6.34	1.9%	8.57	2.6%

3.3 Lot-to-Lot Reproducibility:

Four human sera were measured with two replicates and two runs per day for 10 days (n= 40) using three lots of reagents at Laboratory 1 of KRISHGEN BIOSYSTEMS. The human sera were pooled patient and single donor spiked samples. For Lot 1, the total precision for the four samples ranged from 3.4%–8.3% CV. For Lot 2, the total precision for the four samples ranged from 5.2%–8.6% CV. For Lot3, the total precision for the four samples ranged from 3.3%–4.7%. The combined lot-to-lot reproducibility parameters are detailed in the table below. All values met the manufacturer's acceptance criteria.

Sample	Mean	Within-Run		Between-Run		Between-Day		Between-Lots		Total	
		SD	CV	SD	CV	SD	CV	SD	CV	SD	CV
1	3.06	0.13	4.1%	0.01	0.4%	0.18	6.0%	0.08	2.7%	0.24	7.8%
2	14.98	0.39	2.6%	0.00	0.0%	0.51	3.4%	0.44	2.9%	0.77	5.2%
3	35.4	1.00	2.8%	0.00	0.0%	1.44	4.1%	0.63	1.8%	1.87	5.3%
4	2418	71.0	2.9%	0.00	0.0%	88.7	3.7%	34.0	1.4%	118	4.9%

3.4. Linearity/Assay Reportable Range:

Linearity: In these studies 15 dilutions were prepared by diluting three human serum samples and three human plasma samples spiked with Bevacizumab down across the lower end of the measuring range. Each dilution was measured in three replicates. Results are summarized in the following table:

Sample	Dilution range (ng/ml)	Slope (95% CI)	Intercept (95% CI)	R^2	%CV Range
Serum Sample 1	1.56 –100	0.95 (0.95–0.95)	0.02 (-0.224–0.271)	0.99	-0.2%–0.9%
Serum Sample 2	1.56 –100	1.00 (1.00–1.01)	0.14 (-0.14–0.42)	0.99	-1.1%–6.7%
Serum Sample 3	1.56 –100	0.89 (0.88–0.89)	0.10 (-0.15–0.35)	0.99	-1.6%–3.0%
Plasma Sample 1	1.56 –100	0.89 (0.88–0.90)	0.00 (-0.39–0.40)	0.99	-3.8%–3.8%
Plasma Sample 2	1.56 –100	0.88 (0.88–0.89)	0.30 (-0.10–0.70)	0.99	-3.5%–1.7%

Sample	Dilution range (ng/ml)	Slope (95% CI)	Intercept (95% CI)	R^2	%CV Range
Plasma Sample 3	1.56 –100	0.88 (0.87–0.89)	0.03 (-0.36–0.43)	0.99	-5.4%–2.7%

The data support linearity from 1.56 –100 ng/ml.

4. Traceability and Stability:

4.1 Traceability:

There are no reference standards for Bevacizumab. The results are reported in ng/mL and the method has been standardized in three Laboratories of KRISHGEN BIOSYSTEMS.

4.2 Kit Stability:

Shelf-Life Stability: A real-time stability study set the shelf-life stability of KRIBIOLISA™ Bevacizumab ELISA.

Open-Vial Stability: The assay reagents can be stored opened at 2–8°C for up to 12 weeks.

5. High Dose Hook Effect:

Hook effect was measured using two human serum samples spiked with Bevacizumab to 110 ng/ml. Each sample was then diluted with Sample Diluent to a level below the upper limit of the analytical measuring range and measured with a single replicate using one lot of reagent. The data demonstrated the assay is not susceptible to Bevacizumab excess up to a concentration of 110 ng/ml.

The high dose hook effect refers to measured levels of antigen displaying a significantly lower absorbance than the actual level present in a sample. This appears when a simultaneous ELISA assay is saturated by a very high concentration of sample antigen binding to all available sites on both the solid phase antibody as well as the detection antibody and thereby preventing the sandwich-formation. The antigen-saturated detection antibodies in solution will be washed off giving a falsely low signal. A “hook” is observed in the curve when data is plotted as a signal versus antigen concentration.

Increasing concentrations >10 ng/ml were assayed as unknowns. **The hook capacity yielding an absorbance reading less than the 100 ng/ml standard was ~ 0.1 mg/ml.**