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KRIBIOLISA™ Faricimab (VABYSMO™) ELISA

REF : KBI1326

Ver 1.2

RUO

Enzyme Immunoassay for the Quantitative Determination of Faricimab in human serum and plasma.

RUO	**For Research Use only**	**REF**	**Catalog Number**
	Store At	**LOT**	**Batch Code**
	Manufactured By		**Biological Risk**
	Expiry Date		**Consult Operating Instructions**

REF KBI1326 **Σ 96 tests**

Introduction:

Faricimab is an IgG1-derived bispecific antibody against VEGF-A and Ang-2 for the treatment of age-related macular degeneration and diabetic macular edema. Faricimab is a bispecific antibody that binds to and inhibits both vascular endothelial growth factor (VEGF)-A and angiopoietin-2 (Ang-2). Faricimab was approved by the FDA on January 28, 2022, and is currently marketed under the trademark VABYSMO by Genentech, Inc. It received subsequent approval for the same indications in Canada in May 2022. In July 2022, the EMA's Committee for Medicinal Products for Human Use (CHMP) recommended faricimab be granted marketing authorization for the treatment of neovascular age-related macular degeneration and diabetic macular edema.

The VEGFs are all secreted proteins. VEGF-A121 and VEGF-A165 are secreted as covalently linked homodimeric proteins, whereas the larger isoforms, VEGF-A189 and VEGF-A206, although believed to be secreted, are not readily diffusible and may remain sequestered in the extracellular matrix. VEGF bioavailability may be regulated by plasmin-mediated proteolysis in the carboxy-terminal domains of the larger matrix-bound VEGF isoforms, such as VEGF-A189, to release more diffusible, biologically active species. Human VEGF-A165, the most abundant and biologically active form, is glycosylated at Asn74 and is typically expressed as a 46 kDa homodimer of 23 kDa subunits. VEGF-A121 has biological activity in endothelial cells, but has lower potency than VEGF-A165.

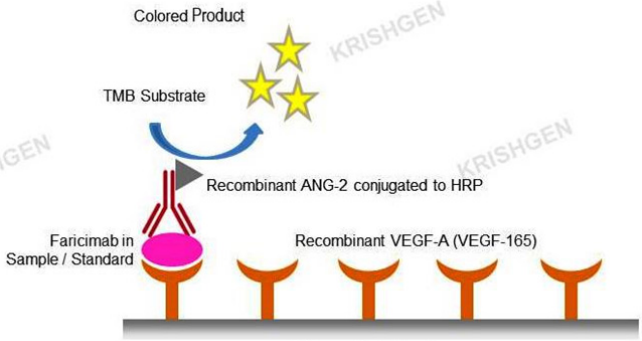
The KRIBIOLISA™ Faricimab (VABYSMO™) ELISA uses VEGF-165 as the capture protein in a sandwich ELISA technique with an HRP conjugated ANG-2 protein as the detection protein to ensure a high degree of specifcitiy to Faricimab.

Intended Use:

The KRIBIOLISA™ Faricimab (VABYSMO™) ELISA is used as an analytical tool for quantitative determination of Faricimab in human serum and plasma.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. VEGF165 protein are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Faricimab present in the sample are bound by the capture protein. Then, a HRP (horseradish peroxidase) conjugated Angiopoietin-2 Protein is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Faricimab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
VEGF165 protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with VEGF-A (VEGF-165) protein.	1 x 96 wells
Faricimab Standard	Recombinant Faricimab in a buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.- (lyophilized, concentrated 1 ug/ml)	2 vials
Angiopoietin-2 protein:HRP Conjugate	Angiopoietin-2 protein conjugated to Horseradish Peroxidase with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml

Part	Description	Qty
(1X) Sample Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane with 1:1000 dilution of human serum	10 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersol < 0.01%. May turn yellow over time.	25 ml
TMB Substrate	Stabilized Chromogen	12 ml
Stop Solution	0.73M Phosphoric Acid	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microtiter Plate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes and multichannel pipettor to measure volumes ranging from 25 ul to 1000 ul
3. Deionized (DI) water
4. Wash bottle or automated microplate washer
5. Standard graph paper or software for data analysis
6. Timer
7. Absorbent Paper

Handling/Storage:

1. All reagents should be stored at 2ºC to 8ºC for stability.
2. All the reagents and wash solutions should be used within 12 months from manufacturing date.
3. Before using, bring all components to room temperature (18-25ºC). Upon assay completion ensure all components of the kit are returned to appropriate storage conditions.
4. The Substrate is light-sensitive and should be protected from direct sunlight or UV sources.

Health Hazard Warnings:

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin.
2. For Research Use Only.

Sample Preparation and Storage:

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Plasma can be used, too. Lipaemic, hemolytic or contaminated samples should not be run. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20°C.

Preparation before Use:

Allow samples to reach room temperature prior to assay. Take care to agitate patient samples gently in order to ensure homogeneity.

Test Sample preparation - Serum and Plasma samples have to be diluted 1:1000 (v/v), e.g. for 1:1000 (1 ul sample + 999 ul Sample Diluent) prior to assay. The samples may be kept at 2 - 8°C for up to three days. Long-term storage requires the samples to be kept at -20°C.

Reagent Preparation (all reagents should be diluted immediately prior to use):

1. Label any aliquots made with the kit Lot No and Expiration date and store it at appropriate conditions mentioned.
2. Bring all reagents to Room Temperature before use.
3. To make **Wash Buffer (1X)**; dilute **25 ml of 20X Wash Buffer in 475 ml of DI water**.

4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 1 ml of Standard Diluent (1X) to obtain a concentration of 1ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 10 ul of reconstituted **Standard (1 ug/ml)** with 990 ul of Standard Diluent (1X) to generate a **10 ng/ml Standard Solution**. Prepare further **Standards** by serially diluting the Standard Solution as per the below table. Use the Standard Diluent (1X) as the Zero Standard (Standard No.0).

Standard Concentration	Standard Vial	Dilution Particulars
1 ug/ml	Lyophilized Standard	Lyophilized Standard provided in the Kit + 1ml of Standard Diluent (1X)
10 ng/ml	Standard No.7	5ul Reconstituted Standard (1 ug/ml) + 495 ul Standard Diluent (1X)
8 ng/ml	Standard No.6	8ul Reconstituted Standard (1 ug/ml) + 992 ul Standard Diluent (1X)
6 ng/ml	Standard No.5	750 ul Standard No.6 + 250 ul Standard Diluent (1X)
4 ng/ml	Standard No.4	666.7 ul Standard No.5 + 333.3 ul Standard Diluent (1X)
2 ng/ml	Standard No.3	250 ul Standard No.4 + 250 ul Standard Diluent (1X)
1 ng/ml	Standard No.2	250 ul Standard No.3 + 250 ul Standard Diluent (1X)
0.5 ng/ml	Standard No. 1	250 ul Standard No.2 + 250 ul Standard Diluent (1X)
0 ng/ml	Standard No.0	Only Standard Diluent (1X)

Procedural Notes:

1. In order to achieve good assay reproducibility and sensitivity, proper washing of the plates to remove excess un-reacted reagents is essential.
2. High Dose Hook Effect may be observed in samples with very high concentrations of Faricimab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Faricimab present in the sample.
3. Avoid assay of Samples containing sodium azide (NaN_3), as it could destroy the HRP activity resulting in under-estimation of the amount of Faricimab.
4. It is recommended that all Standards and Samples be assayed in duplicates.
5. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
6. If the Substrate has a distinct blue color prior to use it may have been contaminated and use of such substrate can lead to compromisation of the sensitivity of the assay.
7. The plates should be read within 30 minutes after adding the Stop Solution.
8. Make a work list in order to identify the location of Standards and Samples.

Assay Procedure:

1. It is strongly recommended that all Standards and Samples be run in duplicates or triplicates. A standard curve is required for each assay. All steps must be performed at 37°C

2. Add **100 ul** of **prepared Standards or diluted Samples** into the respective wells.

3. Cover the plate and incubate for 60 minutes at 37°C

4. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

5. Add **100 ul** of **Angiopoietin-2:HRP Conjugate** into each well.

6. Cover the plate and incubate for 60 minutes at 37°C

7. Aspirate and wash plate 4 times with **Wash Buffer (1X)** and blot residual buffer by firmly tapping plate upside down on absorbent paper. Wipe of any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step.

8. Add **100 ul** of **TMB Substrate** in each well.

9. Incubate the plate at 37°C for 30 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

10. Pipette out **100 ul** of **Stop Solution**. Wells should turn from blue to yellow in color.

11. Read the absorbance at 450 nm with a microplate reader.

Calculation of Results:

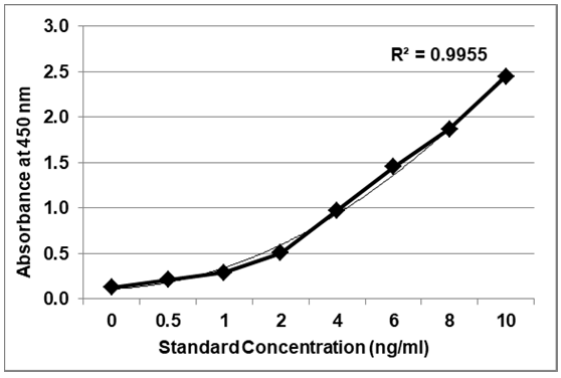
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Faricimab concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Faricimab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or 4PL is best recommended for automated results.

Note:
It is recommended to repeat the assay at a different dilution factor in the following cases:
- If the sample absorbance value is below the first standard.
- If the absorbance value is equivalent or higher than the 10 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Absorbance A	Absorbance B	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.116	0.137	0.127	0.0	--
0.5	0.210	0.216	0.213	0.5	104.8
1	0.277	0.306	0.292	0.9	93.5
2	0.494	0.524	0.509	2.0	99.2
4	0.966	0.986	0.976	4.1	101.7
6	1.446	1.466	1.456	6.1	101.6
8	1.817	1.923	1.870	7.8	97.3
10	2.437	2.465	2.451	10.1	100.9

Typical Graph



Abs = absorbance at 450nm

Quality Control:

It is recommended that for each laboratory assay appropriate quality control samples in each run to be used to ensure that all reagents and procedures are correct.

Performance Characteristics of the Kit:

This kit has been validated as per EMA/FDA guidelines in line with ICH Code for Harmonization of Biological Assays.

Sensitivity:

Limit of Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ was found to be 0.3 ng/ml.

Limit Of Detection: It is defined as the lowest detectable concentration corresponding to a signal of Mean of '0' standard plus 2* SD.
10 replicates of '0' standards were evaluated and the LOD was 0.2 ng/ml.

Specificity:
The recombinant proteins used in the kit are specific for Faricimab bispecific antibody using a sandwich ELISA technique with VEGF-165 (the most common and dominant isoform of VEGF-A) as the capture protein and ANG-2 protein as the detection protein.

Precision:
Precision is defined as the percent coefficient of variation (%CV) i.e. standard deviation divided by the mean and multiplied by 100. Assay precision was determined by both intra (n=5 assays) and inter assay (n=5 assays) reproducibility on two pools with low (0.5 ng/ml), medium (4 ng/ml) and high (10 ng/ml) concentrations. While actual precision may vary from laboratory to laboratory and technician to technician, it is recommended that all operators achieve precision below these design goals before reporting results.

Pool	Intra Assay %CV	Inter Assay %CV
Low	<10%	<10%
Medium	<8%	<8%
High	<5%	<5%

Safety Precautions:

- This kit is **For Research Use only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid time shift during pipetting of reagents.
- All reagents should be kept in the original shipping container.
- Some of the reagents contain small amount of sodium azide (< 0.1 % w/w) as preservative. They must not be swallowed or allowed to come into contact with skin or mucosa.
- Source materials maybe derived from human body fluids or organs used in the preparation of this kit were tested and found negative for HBsAg and HIV as well as for HCV antibodies. However, no known test guarantees the absence of such viral agents. Therefore, handle all components and all patient samples as if potentially hazardous.
- Since the kit contains potentially hazardous materials, the following precautions should be observed
  - Do not smoke, eat or drink while handling kit material
  - Always use protective gloves
  - Never pipette material by mouth
  - Wipe up spills promptly, washing the affected surface thoroughly with a decontaminant.
- In any case GLP should be applied with all general and individual regulations to the use of this kit.

References:

Development and evaluation of an ultrasensitive free VEGF-A immunoassay for analysis of human aqueous humor
JC Göpfert, A Reiser, VA Carcamo Yañez, A Pohle… - Bioanalysis, 2019 - Future Science

Systemic counterregulatory response of angiopoietin-2 after aflibercept therapy for nAMD: a potential escape mechanism
R Angermann, T Rauchegger… - Acta …, 2021 - Wiley Online Library

Aflibercept more effectively weans patients with neovascular age-related macular degeneration off therapy compared with bevacizumab
X Cao, JC Sanchez, TP Patel, Z Yang… - The Journal of …, 2023 - Am Soc Clin Investig

SCHEMATIC ASSAY PROCEDURE

1. Remove all components, 30 minutes before adding into the assay plate.

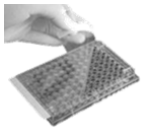
Components (2-8ºC) → 30 mins RT Before Use → 

Thaw at Room Temperature (18-24ºC) → USE NOW

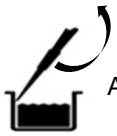
2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

Kit Components → AVOID → COOL (2-8ºC) → THAW (22±4ºC) → THAW (22±4ºC) → COOL (2-8ºC) → AVOID → NO CHANGE IN RESULTS

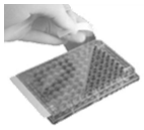
3.  Pipette **100 ul prepared Standards / diluted Samples** into the respective wells.

4. Cover plate  and incubate for 

at 37°C.

5.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**

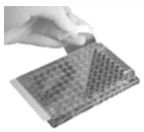
6.  Pipette **100 ul Angiopoietin-2:HRP** into each well.

7. Cover plate  and incubate for 

at 37°C

8.  Aspirate and wash wells 4 times with **Wash Buffer (1X).**

9.  Pipette **100 ul TMB Substrate** into each well.

10. Cover plate  and incubate for 

at 37°C.

11.  Pipette **100 ul Stop Solution** into each well.

12. Read absorbance at 450nm with a  microplate reader within 

of stopping reaction.

LIMITED WARRANTY

Krishgen Biosystems does not warrant against damages or defects arising in shipping or handling, or out of accident or improper or abnormal use of the Products; against defects in products or components not manufactured by Krishgen Biosystems, or against damages resulting from such non-Krishgen Biosystems made products or components. Krishgen Biosystems passes on to customer the warranty it received (if any) from the maker thereof of such non Krishgen made products or components. This warranty also does not apply to Products to which changes or modifications have been made or attempted by persons other than pursuant to written authorization by Krishgen Biosystems.

THIS WARRANTY IS EXCLUSIVE. The sole and exclusive obligation of Krishgen Biosystems shall be to repair or replace the defective Products in the manner and for the period provided above. Krishgen Biosystems shall not have any other obligation with respect to the Products or any part thereof, whether based on contract, tort, and strict liability or otherwise. Under no circumstances, whether based on this Limited Warranty or otherwise, shall Krishgen Biosystems be liable for incidental, special, or consequential damages.

This Limited Warranty states the entire obligation of Krishgen Biosystems with respect to the Products. If any part of this Limited Warranty is determined to be void or illegal, the remainder shall remain in full force and effect.

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SYMBOLS KEY

MTP	VEGF165 protein Coated Microtiter Plate (12x8 wells)
STD	Faricimab Standard
HRP CONJ	Conjugate Horseradish Peroxidase
1X SAMP DIL	(1X) Sample Diluent
1X STD DIL	(1X) Standard Diluent
20X WASH BUF	(20X) Wash Buffer
SUB TMB	TMB Substrate
SOLN STOP	Stop Solution
	Consult Instructions for Use
REF	Catalog Number
	Expiration Date
	Storage Temperature

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