KRIBIOLISA™ Evolocumab (REPATHA™) ELISA

REF : KBI1323

Ver 1.0

RUO

Enzyme Immunoassay for the Quantitative Determination of Evolocumab 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**

For Research Use Only. Purchase does not include or carry the right to resell or transfer this product either as a stand-alone product or as a component of another product. Any use of this product other than the permitted use without the express written authorization of KRISHGEN BioSystems is strictly prohibited.

REF KBI1323 **Σ 96 tests**

KRIBIOLISA™ Evolocumab (REPATHA) ELISA

Email: sales1@krishgen.com

Introduction:

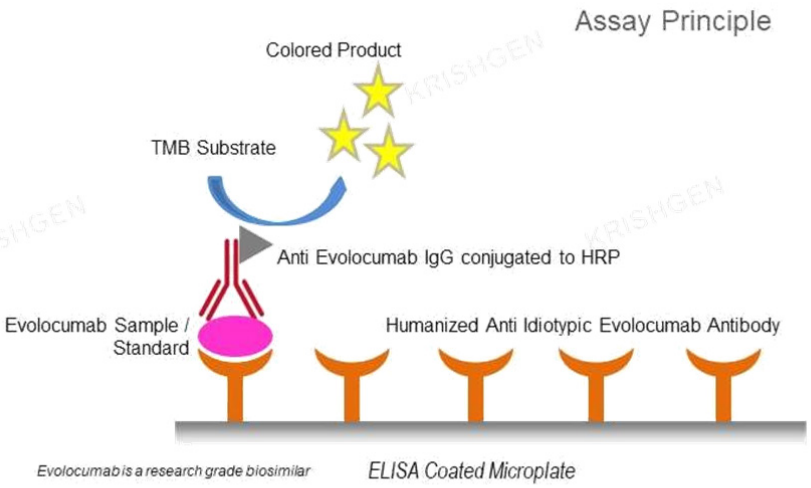
Evolocumab (REPATHA) is a monoclonal antibody medication designed for the treatment of hyperlipidemia.Evolocumab is a fully human monoclonal antibody that inhibits proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 is a protein that targets LDL receptors for degradation; its inhibition thereby enhances the liver's ability to remove LDL-C, often simplistically referred to as "bad" cholesterol, from the blood.Evolocumab is designed to bind to PCSK9 and inhibit PCSK9 from binding to LDL receptors on the liver surface. In the absence of PCSK9, there are more LDL receptors on the surface of liver cells to remove LDL-C from the blood

Intended Use:

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

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Antibodies to Evolocumab are pre-coated onto microwells. Samples and standards are pipetted into microwells and human Evolocumab present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated anti-Evolocumab antibody 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 Evolocumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



PRINCIPLE OF THE KRIBIOLISA™ EVOLOCUMAB (REPATHA) ELISA

Materials Provided:

Part	Description	Qty
Anti- Evolocumab Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-Evolocumab monoclonal antibody.	1 x 96 wells
Evolocumab Standard	Recombinant Evolocumab Buffered protein base with preservative sodium azide < 0.01% (lyophilized, concentrated 1 ug/ml)	2 vials
Anti- Evolocumab:HRP Conjugate concentrated	Anti- Evolocumab conjugated to Horseradish Peroxidase concentrated (1 mg/ml)	1 vial
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml

Part	Description	Qty
(1X) Sample Diluent	Buffered protein base with preservative sodium azide < 0.01%	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with 1:1000 dilution human serum and preservative sodium azide < 0.01%	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 **(1X) 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 to obtain a concentration of 1ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 640 ul of reconstituted **Standard (1 ug/ml)** with 360 ul of Standard Diluent to generate a **640 ng/ml Standard Solution**. Prepare further **Standards** by serially diluting the Standard Solution as per the below table. Use the Standard Diluent 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)
640 ng/ml	Standard No.7	640ul Reconstituted Standard (1 ug/ml) + 360 ul Standard Diluent (1X)
320 ng/ml	Standard No.6	500 ul Standard No.7 + 500 ul Standard Diluent (1X)
160 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
80 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
40 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
20 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
10 ng/ml	Standard No. 1	500 ul Standard No.2 + 500 ul Standard Diluent (1X)
0 ng/ml	Standard No.0	Only Standard Diluent (1X)

5. **Working Anti- Evolocumab:HRP Conjugate** – **Refer to the Reagent Preparation sheet attached with the IFU and COA (enclosed in the kit).**

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 Evolocumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Evolocumab 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 Evolocumab.
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 **Working Anti-Evolocumab: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:

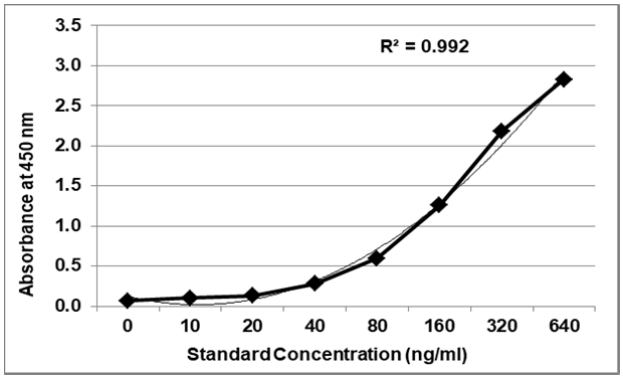
Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Standard graph paper, plot the average value (absorbance 450nm) of each standard on the Y-axis versus the corresponding concentration of the standards on the X-axis. Draw the best fit curve through the standard points. To determine the unknown Evolocumab 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 Evolocumab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or 4PL (2nd order) 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 640 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Abs A	Abs B	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.073	0.065	0.069	---	---
10	0.099	0.109	0.104	11.9	118.8
20	0.146	0.111	0.128	17.7	88.4
40	0.298	0.261	0.279	42.3	105.8
80	0.666	0.523	0.595	80.4	100.5
160	1.397	1.125	1.261	158.0	98.7
320	2.365	2.009	2.187	323.6	101.1
640	2.818	2.844	2.831	635.3	99.3

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 9.50 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 8.05 ng/ml.

Specificity:
The capture antibody is an Anti-Evolocumab Antibody, which is a paratope specific, inhibitory antibody that specifically recognizes the monoclonal antibody drug evolocumab. It does not recognize the drug target, human proprotein convertase subtilisin/kexin type 9 (PCSK9), nor evolocumab in complex with PCSK9. The antibody thus enables a high specificity in measuring free evolocumab in patient samples.

Capture Antibody reactivity. The monovalent intrinsic affinity of the antibody was measured as KD = 1 nM by real time, label free molecular interaction analysis on immobilized evolocumab.

The detection antibody used is a bivalent HRP conjugated antibody which when paired with an anti-evolocumab antibody in monovalent Fab format as the capture antibody ensures a high degree of specificity in this sandwich assay. The standards used in the kit are a monoclonal which detects human PCSK9.

Note: The Standards / Calibrators have not yet been referenced and calibrated against the reference therapeutic drug. Incase of any technical difficutly when using the drug as standard, please connect with our technical team at email: sales1@krishgen.com for further optimization.

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 (10 ng/ml), medium (80 ng/ml) and high (640 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	<15%	<12%
Medium	<12%	<10%
High	<10%	<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 validation of indirect and generic immunoassays to quantify free and total evolocumab in rat serum
W Wang, L Dong, H Sun, L Zhu, W Liu
- Bioanalysis, 2019 - Taylor & Francis

Evolocumab, a proprotein convertase subtilisin/kexin type 9 inhibitor, promotes angiogenesis in vitro
L Safaeian, G Vaseghi, H Jabari…
- Canadian journal of …, 2019 - cdnsciencepub.com

Effect of evolocumab on lipoprotein (a) and PCSK9 in healthy individuals with elevated lipoprotein (a) level
O Afanasieva, MV Ezhov, E Klesareva…
- Journal of …, 2020 - mdpi.com

PCSK9 inhibition-mediated reduction in Lp (a) with evolocumab: an analysis of 10 clinical trials and the LDL receptor's role [S]
FJ Raal, RP Giugliano, MS Sabatine, MJ Koren…
- Journal of lipid …, 2016 - ASBMB

Clinical pharmacokinetics and pharmacodynamics of evolocumab, a PCSK9 inhibitor
S Kasichayanula, A Grover, MG Emery…
- Clinical …, 2018 - Springer

Evolocumab as an immunomodulator in glioma: A window of opportunity trial evaluating PCSK9 inhibition to enhance surface MHC-I on tumor
K Singh, MW Foster, MJ Violette, KM Hotchkiss… - medRxiv, 2024 - medrxiv.org

Evolocumab loaded Bio-Liposomes for efficient atherosclerosis therapy
Z Li, H Zhu, H Liu, D Liu, J Liu, J Jiang, Y Zhang…
- Journal of …, 2023 - Springer

Factorial effects of evolocumab and atorvastatin on lipoprotein metabolism
GF Watts, DC Chan, R Dent, R Somaratne…
- Circulation, 2017 - Am Heart Assoc

SCHEMATIC ASSAY PROCEDURE

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

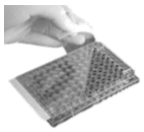
Components (2-8ºC) → 30 mins RT Before Use → 

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

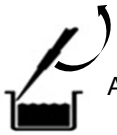
2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

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

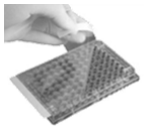
3.  Pipette **100 ul prepared Standards / diluted Samples** into the respective wells.

4. Cover plate  and incubate for 

at 37°C.

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

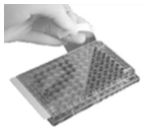
6.  Pipette **100 ul Working Anti- Evolocumab:HRP Conjugate** into each well.

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.

Krishgen Biosystems. 2025

THANK YOU FOR USING KRISHGEN PRODUCT!

KRISHGEN BIOSYSTEMS | OUR REAGENTS | YOUR RESEARCH |

SYMBOLS KEY

MTP	Anti-Evolocumab Coated Microtiter Plate (12x8 wells)
STD	Evolocumab Standard
HRP CONJ	Conjugate Horseradish Peroxidase concentrated
DETN DIL	Detection Diluent
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