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KRIBIOLISA® Dupilumab (DUPIXENT™) ELISA

REF : KBI1279

Ver 2.4

RUO

Enzyme Immunoassay for the quantitative determination of Dupilumab (DUPIXENT™) in human serum

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

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REF KBI1279 Σ 96 tests

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Introduction:

Dupilumab is a fully human monoclonal antibody of the immunoglobulin G4 subclass that binds to the interleukin-4 (IL-4) receptor, inhibiting the receptor signaling pathways. As an interleukin-4 receptor alpha antagonist, dupilumab inhibits the signaling of pro-inflammatory cytokines, called interleukins that induce inflammatory and immunological reactions in several atopic or allergic conditions, such as eczema, allergic reaction, and rhinosinusitis. Dupilumab was generated by recombinant DNA technology in Chinese Hamster Ovary cell suspension culture.

Dupilumab is commonly marketed as Dupixent, which is available as a formulation for subcutaneous injection. It was first approved by the FDA in 2017. It is currently used to treat atopic dermatitis, asthma as an add-on maintenance treatment, chronic rhinosinusitis with nasal polyposis, and eosinophilic esophagitis. It is used as monotherapy or in combination with other drugs, such as corticosteroids. Dupilumab is currently under investigations for potential therapeutic use in diseases driven by allergic reactions or type 2 inflammation, such as pediatric atopic dermatitis, and chronic obstructive pulmonary disease. It is also being studied in combination with another antibody that which targets IL-33.

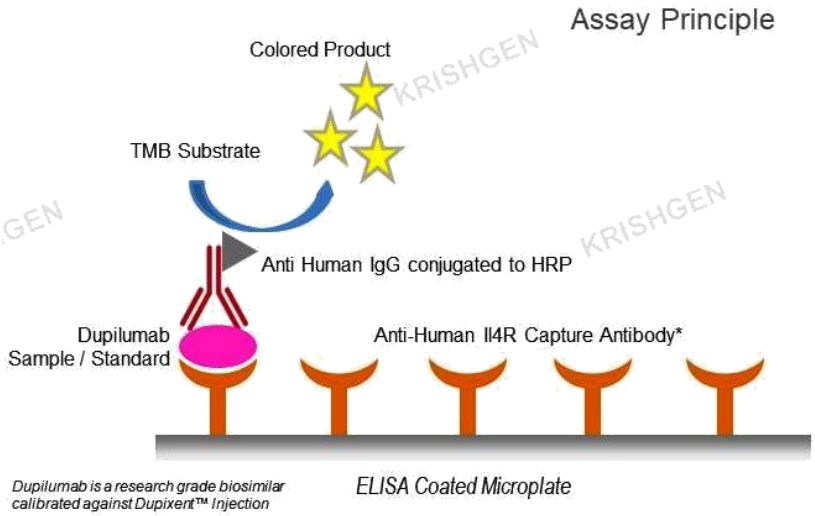
The absorption levels for Dupilumab indicates the C_{max} following administration of a single subcutaneous dose of 600 mg or 400 mg of dupilumab were 70.1 ± 24.1 mcg/ml or 41.8 ± 12.4 mcg/ml, respectively. The Tmax ranged from 3 to 7 days following administration of a single subcutaneous dose ranging from 75 to 600 mg. Following a subcutaneous dose, the absolute bioavailability of dupilumab ranged between 61% and 64% in patients with atopic dermatitis or asthma.

In clinical trials, the steady-state concentrations were reached by week 16 following the administration of 600 mg starting dose and 300 mg dose every other week. At these concentrations, the mean trough concentrations ranged from 60.3 ± 35.1 mcg/ml to 79.9 ± 41.4 mcg/ml for 300 mg dose and from 29.2 ± 18.7 to 36.5 ± 22.2 mcg/ml for 200 mg dose administered every other week.

The KRIBIOLISA® Dupilumab (DUPIXENT™) ELISA has an assay range of 0 - 100 ug/ml and offers dilutional linearity to estimate duplimumab in patient serum.

Intended Use:

The KRIBIOLISA® Dupilumab (DUPIXENT™) ELISA is used as an analytical tool for quantitative determination of Dupilumab (DUPIXENT™) in human serum.



Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Anti-IL4R Antibody is pre-coated onto microwells. Samples and standards are pipetted into microwells and human Dupilumab present in the sample are bound by the capture antibody. Then, a HRP (horseradish peroxidase) conjugated Anti-Human IgG antibody is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use substrate solution 3,3',5,5' Tetra Methyl Benzidine (TMB) is added to microwells and color develops proportionally to the amount of Dupilumab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.

Materials Provided:

Part	Description	Qty
Anti-IL4R Antibody Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Anti-IL4R Antibody	1 x 96 wells
Dupilumab Standard	Recombinant Dupilumab in a buffered protein base with preservative 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane (lyophilized - 100 ug/ml)	2 vials
Anti-Human IgG:HRP Conjugate Concentrated	Anti-Human IgG conjugated to Horseradish Peroxidase Concentrated (1 mg/ml)	2 vials
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane	12 ml
(1X) Sample Diluent	Buffered protein base with preservative 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with 1:1000 dilution human serum and preservative 0.02% Methylisothiazolinone and 0.02% bromonitrodioxane	10 ml
(20X) Wash Buffer	20-fold concentrated solution of buffered surfactant with preservative thiomersal <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. Graph paper or software for data analysis.
6. Timer.
7. Absorbent Paper.

Handling/Storage:

1. It is advisable to aliquot and stores the Anti-Human IgG:HRP Conjugate concentrated at -20°C upon receipt. Rest of the kit components should be stored at 2-8°C. Immediately discard any excess Working Anti-Human IgG:HRP Conjugate after running your assay.
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. 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.
For Cell Culture Supernatant **–** If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80ºC. Avoid repeated freeze-thaw cycles.

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 - Samples have to be diluted **1:1000 (v/v)**, e.g. 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 -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 Standard lyophilized vial with 1 ml of Standard Diluent (1X) to obtain a concentration of 100 ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. 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
100 ug/ml	Reconstituted Standard	Lyophilized Standard provided in the Kit + 1 ml of Standard Diluent (1X)
50 ug/ml	Standard No.7	250 ul Reconstituted Standard + 250 ul Standard Diluent (1X)
25 ug/ml	Standard No.6	250 ul Standard No.7 + 250 ul Standard Diluent (1X)
12.5 ug/ml	Standard No.5	250 ul Standard No.6 + 250 ul Standard Diluent (1X)
6.25 ug/ml	Standard No.4	250 ul Standard No.5 + 250 ul Standard Diluent (1X)
3.125 ug/ml	Standard No.3	250 ul Standard No.4 + 250 ul Standard Diluent (1X)
1.563 ug/ml	Standard No.2	250 ul Standard No.3 + 250 ul Standard Diluent (1X)
0.782 ug/ml	Standard No.1	250 ul Standard No.2 + 250 ul Standard Diluent (1X)
0 ug/ml	Standard No.0	Only Standard Diluent (1X)

Use the Standards immediately upon reconstitution. Discard balance standard after use. Do not store them for further experiments.

5 Working Anti-Human IgG: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 Dupilumab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Dupilumab present in the sample. High Dose Hook effect is most likely encountered from samples early in the purification process. If Hook Effect is possible, the samples to be assayed should be diluted with a compatible diluent. Thus if the Dupilumab concentration of the undiluted sample is less than the diluted sample, this may be indicative of the Hook Effect.
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 Dupilumab.
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. Pipette **100 ul** of prepared **Standards** or diluted **Samples** into the respective wells.

3. Cover the plate and incubate for 90 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-Human IgG: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)** as mentioned in step 4.

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 within 10-15 minutes after addition of Stop solution.

Calculation of Results:

Determine the Mean Absorbance for each set of duplicate or triplicate Standards and Samples. Using Semi-Log graph paper, plot the average value (absorbance 450 nm) 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.

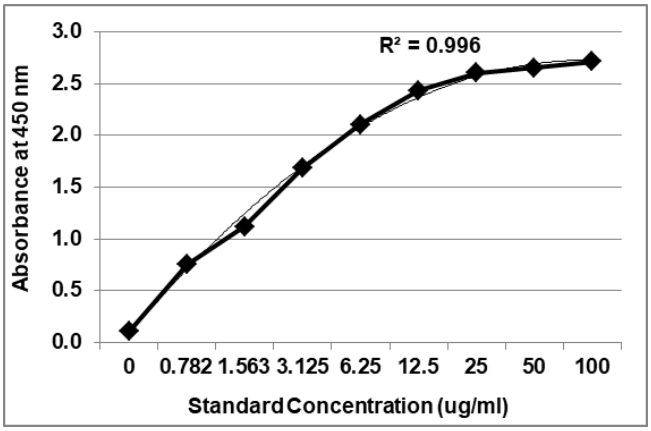
To determine the unknown Dupilumab concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Dupilumab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit 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 100 ug/ml standard.

Typical Data (representative only)

Standard Concentration (ug/ml)	Means Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.109	--	--
0.782	0.755	0.8	105.9
1.563	1.120	1.5	94.5
3.125	1.684	3.2	102.1
6.25	2.102	6.1	97.7
12.5	2.435	13.2	105.9
25	2.602	27.4	109.4
50	2.654	40.7	81.4
100	2.713	94.6	94.6

Typical Graph (representative only)



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 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 found to be less than 0.7 ug/ml.

Specificity:

The kit uses a sandwich format with double antibodies. The capture antibody was produced from a hybridoma resulting from the fusion of a mouse myeloma with B cells obtained from a mouse immunized with purified, recombinant Human IL4R. The detection is Anti-Human IgG monoclonal antibody conjugated to HRP. The standards used in the kit are a biosimilar Duplimumab specific against human CD124/IL4R and receptor identification as IgG4-kappa.

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 (0.782 ug/ml), medium (6.25 ug/ml) and high (100 ug/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	<5%	<5%
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/v) 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:

Wenzel S, Ford L, Pearlman D, Spector S, Sher L, Skobieranda F, Wang L, Kirkesseli S, Rocklin R, Bock B, Hamilton J, Ming JE, Radin A, Stahl N, Yancopoulos GD, Graham N, Pirozzi G: Dupilumab in persistent asthma with elevated eosinophil levels. N Engl J Med. 2013 Jun 27;368(26):2455-66. doi: 10.1056/NEJMoa1304048. Epub 2013 May 21.

Frampton JE, Blair HA: Dupilumab: A Review in Moderate-to-Severe Atopic Dermatitis. Am J Clin Dermatol. 2018 Aug;19(4):617-624. doi: 10.1007/s40257-018-0370-9.

D'Ippolito D, Pisano M: Dupilumab (Dupixent): An Interleukin-4 Receptor Antagonist for Atopic Dermatitis. P T. 2018 Sep;43(9):532-535.

Sastre J, Davila I: Dupilumab: A New Paradigm for the Treatment of Allergic Diseases. J Investig Allergol Clin Immunol. 2018 Jun;28(3):139-150. doi: 10.18176/jiaci.0254.

Davis JD, Bansal A, Hassman D, Akinlade B, Li M, Li Z, Swanson B, Hamilton JD, DiCioccio AT: Evaluation of Potential Disease-Mediated Drug-Drug Interaction in Patients With Moderate-to-Severe Atopic Dermatitis Receiving Dupilumab. Clin Pharmacol Ther. 2018 Dec;104(6):1146-1154. doi: 10.1002/cpt.1058. Epub 2018 Apr 2.

Hurdayal R, Brombacher F: Interleukin-4 Receptor Alpha: From Innate to Adaptive Immunity in Murine Models of Cutaneous Leishmaniasis. Front Immunol. 2017 Nov 10;8:1354. doi: 10.3389/fimmu.2017.01354. eCollection 2017.

FDA Approved Drug Products: Dupixent (dupilumab) for subcutaneous injection

EMA Approved Drug Products: Dupixent (dupilumab) subcutaneous injection

Dupixent® (dupilumab) Approved for Severe Asthma by European Commission - Regeneron

FDA approves first treatment for chronic rhinosinusitis with nasal polyps

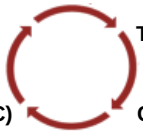
SCHEMATIC ASSAY PROCEDURE

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

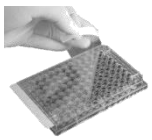
Components (2-8ºC) → 30 mins, RT Before Use → 

→ 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

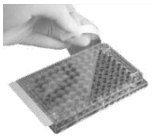
3.  Pipette **100 ul Standards** / diluted **Samples** into each well.

4. Cover plate  and incubate for 

at 37°C.

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

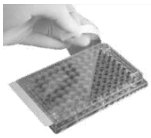
6.  Pipette **100 ul Working Anti-Human IgG:HRP Conjugate** into each well.

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 450 nm with a  microplate reader within 

of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450 nm	Mean Absorbance	ug/ml Dupilumab equivalent
1A 2A	zero std zero std			
1B 2B	0.782 ug/ml 0.782 ug/ml			
1C 2C	1.563 ug/ml 1.563 ug/ml			
1D 2D	3.125 ug/ml 3.125 ug/ml			
1E 2E	6.25 ug/ml 6.25 ug/ml			
1F 2F	12.5 ug/ml 12.5 ug/ml			
1G 2G	25 ug/ml 25 ug/ml			
1H 2H	50 ug/ml 50 ug/ml			
3A 4A	100 ug/ml 100 ug/ml			
3B 4B	Sample			

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Krishgen Biosystems Private Limited 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 Private Limited, or against damages resulting from such non-Krishgen Biosystems Private Limited made products or components. Krishgen Biosystems Private Limited 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 Private Limited.

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This Limited Warranty states the entire obligation of Krishgen Biosystems Private Limited 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	**Anti-IL4R Antibody Coated Microtiter Plate (12x8 wells)**
STD	**Dupilumab Standard, Lyophilized**
HRP CONJ	**Anti-Human IgG:HRP Conjugate concentrated**
DETN DIL	**Detection Diluent**
1X STD DIL	**(1X) Standard Diluent**
1X SAMP DIL	**(1X) Sample 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**