KRIBIOLISA™ Blinatumomab (BLINCYTO) ELISA

REF : KBI1197

Ver 2.0

RUO

Enzyme Immunoassay for the Quantitative Determination of Blinatumomab 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**

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REF KBI1197 **Σ 96 tests**

KRISHGEN BioSystems | For US / Europe: toll free +1(888)-970-0827 tel: +1(562)-568-5005
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Email: sales1@krishgen.com

KRIBIOLISA™ Blinatumomab (BLINCYTO) ELISA

Introduction:

Blinatumomab, marketed under the brand name BLINCYTO, is a monoclonal antibody used in the treatment of certain types of leukemia. It is specifically designed to target and bind to both CD19, a protein found on the surface of B cells, and CD3, a protein on the surface of T cells. Blinatumomab brings these immune cells into close proximity, facilitating the destruction of B cells by the activated T cells. BLINCYTO is approved for the treatment of Philadelphia chromosome-negative relapsed or refractory B-cell precursor acute lymphoblastic leukemia (ALL) in both pediatric and adult patients. This immunotherapy has demonstrated efficacy in patients who have not responded to other treatments or have experienced relapse, offering a novel approach to the management of this aggressive form of leukemia by harnessing the immune system's ability to target and eliminate cancer cells.

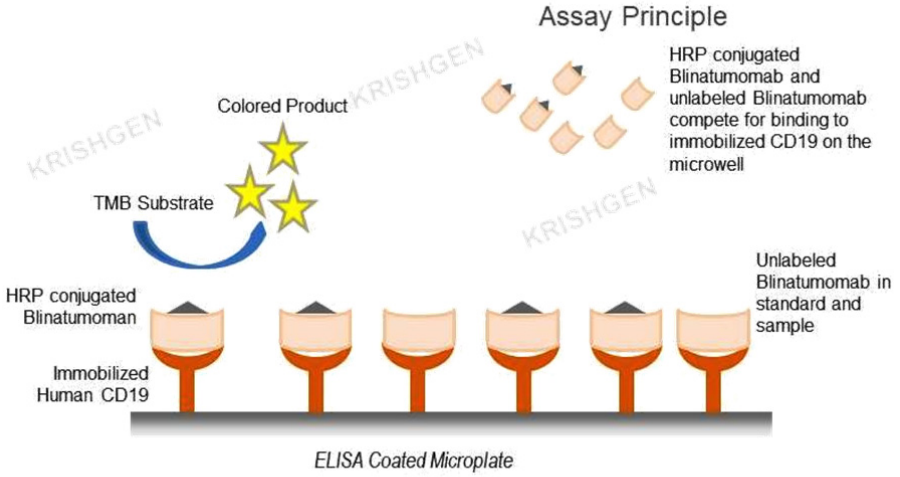
In summary, Blinatumomab is a bispecific T-cell engager (BiTE). It enables a patient's T cells to recognize malignant B cells. A molecule of blinatumomab combines two binding sites: a CD3 site for T cells and a CD19 site for the target B cells.

Intended Use:

The KRIBIOLISA™ Blinatumomab (BLINCYTO) ELISA kit is used for the quantitative estimation of Blinatumomab in cell culture supernatant and pharmaceutical preparations.

Principle:

The Blinatumomab ELISA is a competitive immunoassay for the determination of Blinatumomab. It is known that Blinatumomab binds strongly to Human CD19/Leu-12. Hence using this principle the assay has been developed. A varying concentration of unlabeled standard or sample and constant concentration of Blinatumomab: HRP conjugate will bind in sequence to the Human CD19/Leu-12 protein coated on the microplate. Upon washing, unbound Blinatumomab: HRP Conjugate will be removed. Bound Blinatumomab:HRP complex will produce a soluble blue colored product after the addition of TMB Substrate. The enzyme reaction is stopped by dispensing of stop solution into the wells. The optical density (OD) of the solution at 450 nm is inversely proportional to the amount of bound Blinatumomab present in the standards or samples.



PRINCIPLE OF THE KRIBIOLISA™ BLINATUMOMAB ELISA

Materials Provided:

Part	Description	Qty
Human CD19/Leu-12 protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Human CD19/Leu-12 protein Coated Microtiter Plate	1 x 96 wells
Blinatumomab Standard	Blinatumomab in Buffered protein base with preservative thiomersol < 0.01% standard (lyophilized, concentration - 2,000 ng/ml)	2 vials

Part	Description	Qty
Blinatumomab:HRP Conjugate concentrated	Blinatumomab HRP conjugate (concentrated 1mg/ml)	1 vial
Detection Diluent	Buffered protein base with protein stabilizer and preservatives 0.02% methylisothiazolone and 0.02% bromonitrodioxane.	12 ml
(1X) Sample Diluent	Buffered protein base with preservative thiomersol < 0.01%	50 ml
(1X) Standard Diluent	Buffered protein base with preservative thiomersol < 0.01% and 1:10 dilution 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 TMB Chromogen	12 ml
Stop Solution	0.73M Phosphoric Acid	12 ml
Instruction Manual		1 no

Materials to be provided by the End-User:

1. Microplate Reader able to measure absorbance at 450 nm.
2. Adjustable pipettes to measure volumes ranging from 50 ul to 1000 ul.
3. Deionized (DI) water.
4. Wash bottle or automated microplate washer.
5. Graph paper or software for data analysis.
6. Tubes to prepare standard/sample dilutions.
7. Timer.
8. Absorbent paper.
9. Incubator

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.

For Cell Culture Supernatant **–** If necessary, centrifuge to remove debris prior to analysis. Samples can be stored at -20°C or -80^{0}C. 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 1:10 (v/v), e.g. for 1:10 (10 ul sample + 90 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 **(1X) Wash Buffer**; dilute **25 ml of 20X Wash Buffer in 475 ml of DI water**.
4. **Standards Preparation**: Reconstitute the concentrated Standard lyophilized vial with 250 ul of (1X) Standard Diluent to obtain 8000 ng/ml. Keep the vial for 15 mins with gentle agitation and then run the assay procedure. Use the (1X) Standard Diluent as the zero standard. Below table shows the calculation for the standard range.

Standard Concentration (ng/ml)	Standard No.	Dilution Particulars
2000 ng/ml	Lyophilized	Lyophilized Standard
8000 ng/ml	Reconstituted	Reconstitute in 250 ul Standard Diluent (1X)
6000 ng/ml	Standard No.7	187.5 ul Reconstituted Standard +62.5 ul Standard Diluent (1X)
3000 ng/ml	Standard No.6	125 ul Standard No.7 + 125 ul Standard Diluent (1X)
1500 ng/ml	Standard No.5	125 ul Standard No.6 + 125 ul Standard Diluent (1X)
750 ng/ml	Standard No.4	125 ul Standard No.5 + 125 ul Standard Diluent (1X)
375 ng/ml	Standard No.3	125 ul Standard No.4 + 125 ul Standard Diluent (1X)
187.5 ng/ml	Standard No.2	125 ul Standard No.3 + 125 ul Standard Diluent (1X)
93.75 ng/ml	Standard No.1.	125 ul Standard No.2 + 125 ul Standard Diluent (1X)
0 ng/ml	Standard No.0	250 ul Standard Diluent (1X)

Mix each tube thoroughly before the next transfer. Use the standards for experiment within one hour of preparation of standard. Discard standard after use.

5. **Working Blinatumomab: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. Avoid assay of Samples containing Sodium Azide (NaN_3), as it could destroy the HRP activity of the conjugate resulting in under-estimation of the antibodies.
3. It is recommended that all Standards and Samples be assayed in duplicates or triplicates.
4. Maintain a repetitive timing sequence from well to well for all the steps to ensure that the incubation timings are same for each well.
5. 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.
6. The plates should be read within 30 minutes after adding the Stop Solution.
7. Make a work list in order to identify the location of Standards and Samples.
8. Making serial dilution in the wells directly is not permitted.
9. Prepare the Standard within 15 minutes prior to running the assay.
10. Please carefully dilute Standards according to the instruction, and avoid foaming. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettes are calibrated.
11. If crystals have formed in the Wash Solution (20X) concentrate, warm to room temperature and mix gently until the crystals are completely dissolved.
12. Contaminated water or container for reagent preparation will influence the detection results.

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.

2. Pipette out **100 ul of Standards and samples** to the respective wells.

3. Add **200 ul working Blinatumomab: HRP Conjugate** to each well.

4. Cover the plate and incubate for 210 mins at 37°C.

5. 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.

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

7. Incubate the plate at RT 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.

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

9. 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 Semi-Log 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 Blinatumomab 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 Blinatumomab Concentration.

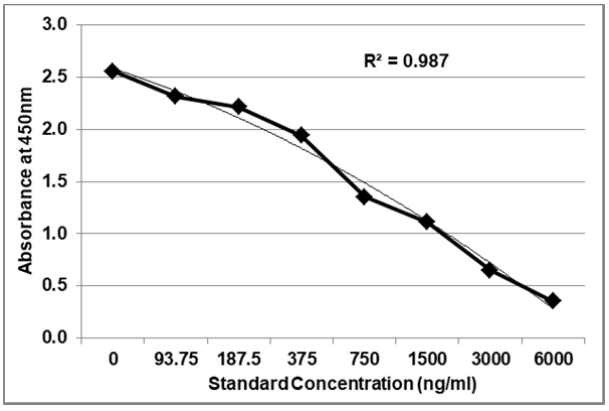
If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or 4PL (2nd order) is best recommended for automated results.

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 6000 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0.0	2.555	--	--
93.75	2.313	72.1	115.3
187.5	2.213	109.9	87.9
375.0	1.942	221.0	88.4
750.0	1.353	609.7	121.9
1500.0	1.110	888.0	88.8
3000.0	0.649	1986.9	99.3
6000.0	0.356	4194.5	104.9

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.

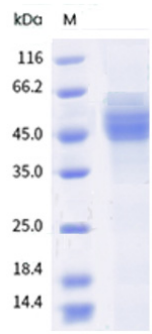
Performance Characteristics:

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 and the dilutional linearity 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.

Standard Range:
93.75 ng/ml - 6000 ng/ml.

Specificity:
The capture protein is specific to Blinatumomab/Anti-CD19. It is expressed in mammalian cells with a protein construct using a DNA sequence encoding the human CD19 (P15391-1) extracellular domain (Met1-Lys291).



The recombinant human CD19 consists of 283 amino acids and predicts a molecular mass of 31.6 kDa. In SDS-PAGE under reducing conditions, rhCD19 migrates as an approximately 47 kDa band due to glycosylation.

The detection conjugate and labeled antibody is specific to Blinatumomab biosimilar, research grade. It is a monoclonal antibody expressed in CHO cells and targets CD19/CD3.

Sensitivity:
The minimum detectable dose of Blinatumomab is 90 ng/ml.

Precision:
Intra-Assay Precision: 3 samples with low, middle and high level human Blinatumomab were tested 20 times on one plate, respectively.
Inter-Assay Precision: 3 samples with low, middle and high level human Blinatumomab were tested on 3 different plates, 8 replicates in each plate.

CV (%) = SD/mean X 100
Intra-Assay: CV<15%
Inter-Assay: CV<18%

Safety Precautions:

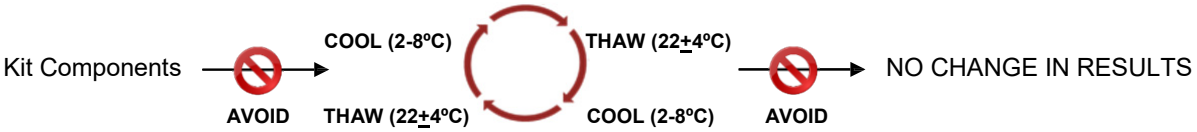
- **This kit is For Research Use only**. Follow the working instructions carefully.
- The expiration dates stated on the kit are to be observed. The same relates to the stability stated for reagents
- Do not use or mix reagents from different lots.
- Do not use reagents from other manufacturers.
- Avoid 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.

SCHEMATIC ASSAY PROCEDURE

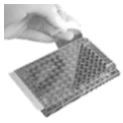
1. Remove all components, 30 minutes before adding into the assay plate.

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



3.  Pipette **100 ul prepared Standard** and **Sample** into respective well.

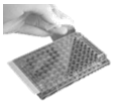
4.  Pipette **200 ul working Blinatumomab:HRP Conjugate** into each wells.

5. Cover plate  and incubate for 

at 37°C.

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

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

8. Cover plate  and incubate for 

at Room Temperature.

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

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

of stopping reaction.

Typical Example of a Work List

Well #	Contents	Absorbance at 450nm	Mean Absorbance	ng/ml Blinatumomab
1A 2A	zero standard zero standard			
1B 2B	62.5 ng/ml 62.5 ng/ml			
1C 2C	125 ng/ml 125 ng/ml			
1D 2D	250 ng/ml 250 ng/ml			
1E 2E	500 ng/ml 500 ng/ml			
1F 2F	1000 ng/ml 1000 ng/ml			
1G 2G	2000 ng/ml 2000 ng/ml			
1H 2H	4000 ng/ml 4000 ng/ml			
3A 4A	Sample Sample			
3B 4B	Sample Sample			
3C 4C	Sample Sample			

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SYMBOLS KEY

MTP	Human CD19/Leu-12 protein Coated Microtiter Plate (12 x 8 wells)
STD	Blinatumomab Standard
HRP CONJ	Blinatumomab:HRP Conjugate concentrated
DET 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