KRISHGEN BioSystems
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KRIBIOLISA® Solitomab ELISA

REF : KBI1767

Ver 1.1

RUO

Enzyme Immunoassay for the Quantitative Determination of Solitomab 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 Private Limited is strictly prohibited.

REF KBI1767 Σ 96 tests

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Introduction:

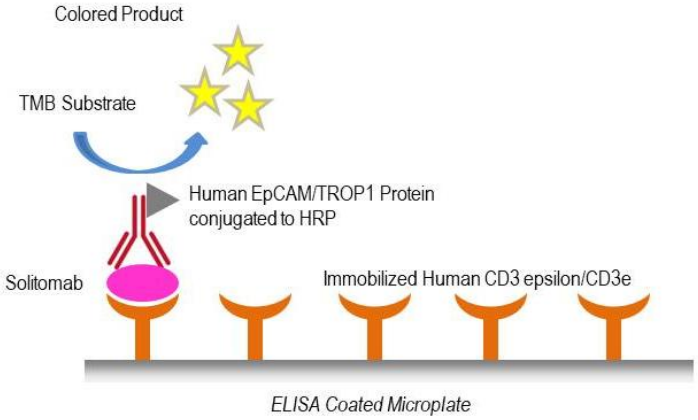
Solitomab is an artificial bispecific monoclonal antibody that is being investigated as an anti-cancer drug. It is a fusion protein consisting of two single-chain variable fragments (scFvs) of different antibodies on a single peptide chain of about 55 kilodaltons. One of the scFvs binds to T cells via the CD3 receptor, and the other to EpCAM as a tumor antigen against gastrointestinal, lung, and other cancers. Solitomab forms a link between T cells and its target tumor cell antigen. This causes T cells to exert cytotoxic activity on tumor cells by producing proteins like perforin and granzymes, independently of the presence of MHC I or co-stimulatory molecules. These proteins enter tumor cells and initiate the cell's apoptosis.This action mimics physiological processes observed during T cell attacks against tumor cells.

Intended Use:

The KRIBIOLISA® Solitomab ELISA is used as an analytical tool for quantitative determination of Solitomab in human serum and plasma.

Principle:

The method employs the quantitative sandwich enzyme immunoassay technique. Human CD3 epsilon protein is pre-coated onto microwells. Samples and standards are pipetted into microwells and human Solitomab present in the sample are bound by the capture protein. Then, a HRP (horseradish peroxidase) conjugated Human EpCAM Protein is pipetted and incubated. After washing microwells in order to remove any non-specific binding, the ready to use Tetra Methyl Benzidine Substrate Solution is added to microwells and color develops proportionally to the amount of Solitomab in the sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm.



Materials Provided:

Part	Description	Qty
Human CD3 epsilon protein Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Human CD3 epsilon protein	1 x 96 wells
Solitomab Standard	Recombinant Solitomab in a buffered protein base with protein stabilizer and preservatives thiomersal <0.01%. (lyophilized, concentrated 16 ug/ml)	2 vials
Human EpCAM protein:HRP Conjugate Concentrated	Human EpCAM protein conjugated to Horseradish Peroxidase Concentrated (1 mg/ml)	1 vial
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 protein stabilizer and preservatives thiomersal <0.01%	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with protein stabilizer and preservatives thiomersal <0.01% with 1:1000 dilution of human serum	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. Standard graph paper or software for data analysis.
6. Timer.
7. Absorbent Paper.

Handling/Storage:

1. It is advisable to aliquot and stores the Human EpCAM protein: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 Human EpCAM protein: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. 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 Standard lyophilized vial with 1 ml of Standard Diluent (1X) to obtain a concentration of 16 ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Prepare further **Standards** by serially diluting the Top Standard (16 ug/ml) as per the below table. Use the Standard Diluent (1X) as the Zero Standard (Standard No. 0).

Standard Concentration	Standard Vial	Dilution Particulars
16000 ng/ml	Reconstituted Standard	Lyophilized Standard provided + 1 ml of Standard Diluent (1X)
8000 ng/ml	Standard No.6	500 ul Reconstituted Standard + 500 ul Standard Diluent (1X)
4000 ng/ml	Standard No.5	500 ul Standard No.6 + 500 ul Standard Diluent (1X)
2000 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent (1X)
1000 ng/ml	Standard No.3	500 ul Standard No.4 + 500 ul Standard Diluent (1X)
500 ng/ml	Standard No.2	500 ul Standard No.3 + 500 ul Standard Diluent (1X)
250 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)

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

5 Working Human EpCAM protein: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 Solitomab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Solitomab 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 Solitomab.
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 180 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 Human EpCAM: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 Standard 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 Solitomab 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 Solitomab Concentration. If samples were

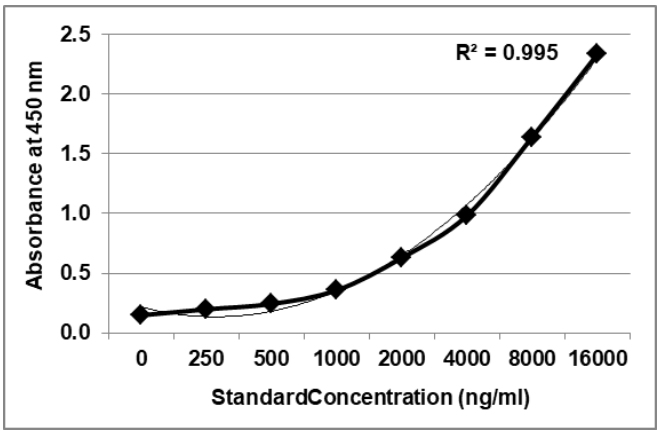
diluted, multiply by the appropriate dilution factor. Software which is able to generate a cubic spline curve-fit or 4PL is best recommended for automated results.

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 (representative only)

Standard Concentration (ng/ml)	Mean Absorbance	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.149	--	--
250	0.199	265.3	106.1
500	0.244	468.1	93.6
1000	0.358	958.6	95.9
2000	0.630	2138.5	106.9
4000	0.984	3849.9	96.2
8000	1.642	8104.4	101.3
16000	2.336	15956.3	99.7

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.

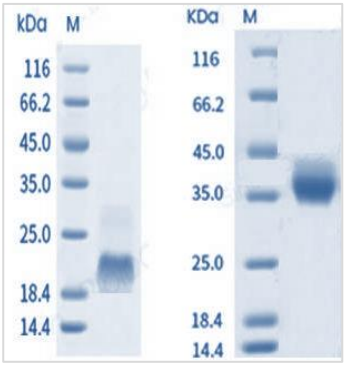
Sensitivity:
Limit of Quantification: It is defined as the lowest concentration of an analyte that can be determined with an acceptable repeatability and the LOQ is ~125 ng/ml.

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 255 ng/ml.

Specificity:

The capture protein Human CD3e protein is HEK293 expressed using a DNA sequence construct encoding the extracellular domain (Met 1-Asp 126) of human CD3E (NP_000724.1) precursor. The purity is >90% by SEC-HPLC. The detection protein is Human EpCAM conjugated to HRP. The detection protein is HEK293 expressed using a DNA sequence construct encoding the extracellular domain (Met1-Lys265) of human EpCAM (NP_002345.1). The purity is >90% by SEC-HPLC. This ensures that there is a high degree of specificity to capture and detect the bispecific Solitomab.

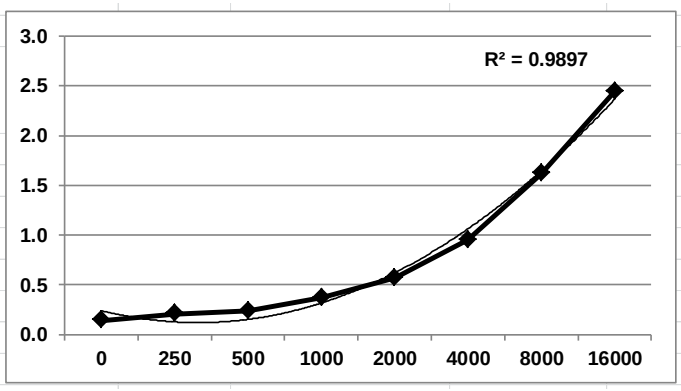
SDS Page of Capture Protein Human CD3e



SDS Page of Detection Protein Human EpCAM

Validation Parameters:

Standard Concentration (ng/ml)	Absorbance 1	Absorbance 2	Mean Absorbance	Interpolated Concentration	% Recovery	Net Signal Difference
0	0.152	0.132	0.142		--	--
250	0.213	0.200	0.206	299.5	119.8	0.061
500	0.241	0.232	0.236	447.5	89.5	0.028
1000	0.387	0.354	0.370	1075.4	107.5	0.146
2000	0.580	0.556	0.568	2002.1	100.1	0.193
4000	0.991	0.905	0.948	3912.1	97.8	0.412
8000	1.712	1.524	1.618	8081.8	101.0	0.721
16000	2.489	2.401	2.445	15967.5	99.8	0.777



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 (250 ng/ml), medium (2000 ng/ml) and high (16000 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%

Run 1

Standard (ng/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Mean Absorbance	% Standard Deviation	% CV
250	0.199	0.185	0.188	0.191	0.6	3.8
2000	0.472	0.433	0.417	0.441	2.3	6.5
8000	1.144	1.141	1.140	1.142	0.2	0.2

Run 2

Standard (ng/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Mean Absorbance	% Standard Deviation	% CV
250	0.159	0.148	0.147	0.151	0.5	4.3
2000	0.400	0.373	0.407	0.393	1.5	4.6
8000	1.164	1.004	1.102	1.090	6.6	7.4

Run 3

Standard (ng/ml)	Absorbance 1	Absorbance 2	Absorbance 3	Mean Absorbance	% Standard Deviation	% CV
250	0.153	0.155	0.154	0.154	0.1	0.7
2000	0.484	0.393	0.493	0.457	4.5	12.1
8000	1.042	0.923	1.045	1.003	5.68	6.9

Parallelism

Known concencentration of the sample (solitomab) was spiked in human serum diluted to 1:1000 using the kit Diluent. Subsequently further dilutions were done to 1:2, 1:4, 1:16, 1:32 and 1:64 and recovery obtained. The kit passes the test for parallelism with the mean recvoeries within the parameter of 80-120% of the known concentrations.

	Expected Standard Conc (ng/ml)	Abs1	Abs2	Mean Abs	Interpolated Conc (ng/ml)	% Recovery	Dilution Factor	Final Conc (ng/ml)	Final Conc (ug/ml)	Expected Conc (ug/ml)	% Recovery
1:2000 dilution	16000	2.144	2.348	2.246	13648.8	85.3	2.0	27297.7	27.3	32.00	117.2
1:4000 dilution	8000	1.668	1.586	1.627	8147.7	101.8	4.0	32590.6	32.6	32.00	98.2
1:8000 dilution	4000	1.086	1.090	1.088	4682.9	117.1	8.0	37463.0	37.5	32.00	85.4
1:16000 dilution	2000	0.669	0.609	0.639	2342.8	117.1	16.0	37484.5	37.5	32.00	85.4
1:32000 dilution	1000	0.445	0.451	0.448	1437.7	143.8	32.0	46007.3	46.0	32.00	69.6
1:64000 dilution	500	0.359	0.323	0.341	940.8	188.2	64.0	60211.7	60.2	32.00	53.1

Abs = absorbance at 450nm; conc = concentration

Matrix Effect Study:

Prepared standards were diluted in the kit Diluent and a second set of prepared standards were diluted in the Kit diluent with 1:1000 normal human serum. The kit recommends using 1:1000 serum dilution to ensure optimal results.

	Kit Diluent	Kit Diluent + 1:1000 Normal Human Serum		
Standard Concentration (ng/ml)	**Mean Absorbance**	**Mean Absorbance**	**% Standard deviation**	**% CV**
0	0.142	0.142	0.0	0.3
250	0.254	0.206	3.4	14.6
500	0.336	0.236	7.1	24.7
1000	0.562	0.370	13.6	29.1
2000	0.822	0.568	18.0	25.9
4000	1.329	0.948	26.9	23.6
8000	2.107	1.618	34.5	18.5
16000	2.945	2.445	35.4	13.1

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.

SCHEMATIC ASSAY PROCEDURE

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

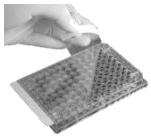
Components (2-8ºC) → 30 mins RT Before Use → 

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

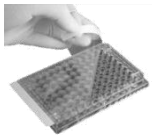
3.  Pipette **100 ul prepared Standards / diluted Samples** into the respective wells.

4. Cover plate  and incubate 

at 37°C.

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

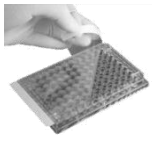
6.  Pipette **100 ul Working Human EpCAM:HRP Conjugate** into each well.

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 450nm	Mean Absorbance	ng/ml Solitomab equivalent
1A 2A	zero std zero std			
1B 2B	250 ng/ml 250 ng/ml			
1C 2C	500 ng/ml 500 ng/ml			
1D 2D	1000 ng/ml 1000 ng/ml			
1E 2E	2000 ng/ml 2000 ng/ml			
1F 2F	4000 ng/ml 4000 ng/ml			
1G 2G	8000 ng/ml 8000 ng/ml			
1H 2H	16000 ng/ml 16000 ng/ml			
3A 4A	Sample Sample			
3B 4B	Sample Sample			

LIMITED WARRANTY

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	**Human CD3 epsilon protein Coated Microtiter Plate (12x8 wells)**
STD	**Solitomab Standard, Lyophilized**
HRP CONJ	**Human EpCAM protein:HRP Conjugate, 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**