KRIBIOLISA™ Anti-Cetuximab (ERBITUX™) ELISA

REF : KBI2018

Ver 2.1

RUO

Enzyme Immunoassay for the Quantitative Determination of Antibodies to Anti-Cetuximab 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 KBI2018 Σ 96 tests

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Introduction:

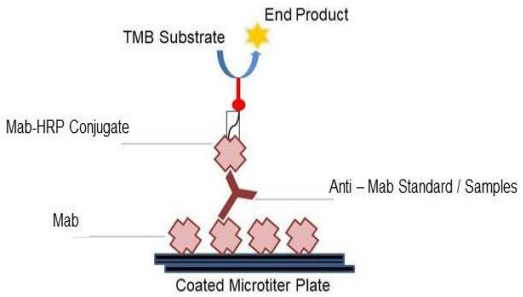
Cetuximab is an epidermal growth factor receptor (EGFR) inhibitor used for the treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer and head and neck cancer. Cetuximab is a chimeric (mouse/human) monoclonal antibody given by intravenous infusion that is distributed under the trade name in the U.S. and Canada by the drug company Bristol-Myers Squibb and outside the U.S. and Canada by the drug company Merck KGaA. In Japan, Merck KGaA, Bristol-Myers Squibb and Eli Lilly have a co-distribution. In July 2009, the FDA approved Cetuximab (Erbitux™) for treatment of colon cancer with wild-type KRAS, since it had little or no effect in colorectal tumors harboring a KRAS mutation (this also applied to the EGFR antibody panitumumab).

Intended Use:

This KRIBIOLISA™ Anti-Cetuximab ELISA is used as an analytical tool for determination of antibodies to Anti-Cetuximab in human serum and plasma.

Principle:

The method employs the quantitative enzyme immunoassay technique. Erbitux™ and Proposed Biosimilar Cetuximab are pre-coated onto microwells. Human Anti-Cetuximab standard or samples are added to microwells. After incubation and washing, Cetuximab-HRP Conjugate is pipetted into microwells. An incubation followed by washing cycle, the ready to use substrate solution (TMB) is added to microwells and color develops proportionally to the amount of Anti-Cetuximab in Standard or in sample. Color development is then stopped by addition of stop solution. Absorbance is measured at 450 nm



Materials Provided:

Part	Description	Qty
Erbitux™ Cetuximab Coated Microtiter Plate	96 well polystyrene microplate (12 strips of 8 wells) coated with Erbitux™ Cetuximab	1 x 96 wells
Human Anti-Cetuximab Standard	Recombinant Anti-Cetuximab in a buffered protein base with preservative sodium azide - lyophilized (1 ug/ml)	2 vials
Cetuximab:HRP Conjugate	Cetuximab:HRP Conjugate prepared in buffer 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%	2 x 50 ml
(1X) Standard Diluent	Buffered protein base with preservative thiomersol < 0.01% with 1:1000 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 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. Store main kit components at 2-8°C
2. Store the recombinant standard at- 20^{0}C. Avoid repeated freeze-thaw cycles.
3. Before using, bring all components to room temperature (18-25°C). Upon assay completion return all components 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.

Sample Preparation and Storage:

Specimens should be clear and non-hemolyzed. Samples should be run at a number of dilutions to ensure accurate quantitation.

Blood is taken by venipuncture. Serum is separated after clotting by centrifugation. Repeated freezing and thawing should be avoided. If samples are to be used for several assays, initially aliquot samples and keep at - 20°C.

Serum and Plasma Samples should be diluted 1:1000 (v/v) for optimal recovery, (for example 1 ul sample + 999 ul sample diluent) prior to assay. In cases where matrix interferences is under or over observed, the samples may be diluted with Sample Diluent accordingly.

The samples may be kept at 2 - 8°C for up to three days. For long-term storage please store at -20°C.

Note: *Grossly hemolyzed samples are not suitable for use in this assay*

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 1 ug/ml. Keep the vial for 15 mins with gentle agitation before making further dilutions. Dilute 640 ul of original **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
1000 ng/ml	Lyophilized	Lyophilized Standard + Reconstitute in 1ml Standard Diluent
640 ng/ml	Standard No.5	640 ul Reconstituted Standard + 360 ul Standard Diluent
320 ng/ml	Standard No.4	500 ul Standard No.5 + 500 ul Standard Diluent
240 ng/ml	Standard No.3	750 ul Standard No.4 + 250 ul Standard Diluent
160 ng/ml	Standard No.2	666.7 ul Standard No.3 + 333.3 ul Standard Diluent
80 ng/ml	Standard No.1	500 ul Standard No.2 + 500 ul Standard Diluent
0 ng/ml	Standard No. 0	Only Standard Diluent

Use the Standards as soon as possible upon dilution. Discard balance standard after use.

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 resulting in under-estimation of the amount of Anti-Cetuximab
3. It is recommended that the Standards and Samples be assayed in duplicates.
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.

Assay Procedure:

1. Pipette **100 ul** of **Standards** and **diluted Samples** to the respective wells.

2. Seal plate and incubate for 1 hour at 37°C.

3. 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. All the washes should be performed similarly.

4. Add **100 ul** of **Cetuximab: HRP Conjugate** to each well.

5. Seal plate and incubate for 1 hour at 37°C.

6. 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. All the washes should be performed similarly.

7. Pipette **100 ul** of **TMB Substrate solution** in all wells.

8. Incubate in the dark for 30 minutes at 37°C.

9. Stop reaction by adding **100 ul** of **Stop Solution** to each well.

10. Read absorbance at 450 nm within 30 minutes of stopping reaction.

Interpretation 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.

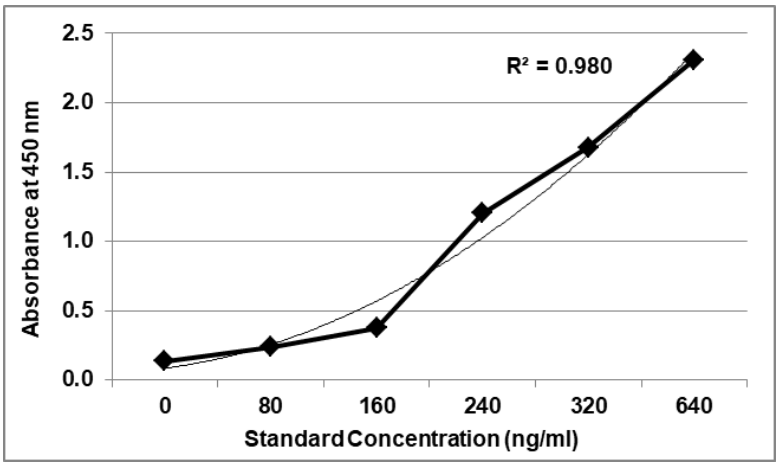
To determine the unknown Anti-Cetuximab concentrations, find the unknown's Mean Absorbance value on the Y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the X-axis and read the Anti-Cetuximab Concentration. If samples were diluted, multiply by the appropriate dilution factor. Software which is able to generate a polynomial regression (2^{nd} order), 4PL or 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 640 ng/ml standard.

Typical Data

Standard Concentration (ng/ml)	Mean Abs	Interpolated Concentration	% Interpolated Concentration against Actual Concentration
0	0.136	--	--
80	0.236	87.5	109.4
160	0.376	142.8	89.3
240	1.202	255.7	106.5
320	1.676	314.0	98.1
640	2.308	640.2	100.0

Typical Graph



Abs= absorbance @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:

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 70 ng/ml

Linearity:
Standards provided in the kit were used for measuring the linearity range of Anti-Cetuximab present in serum and plasma.

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. 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%	<15%
Medium	<12%	<12%
High	<12%	<12%

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.

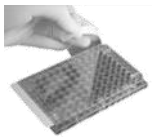
Components (2-8ºC) → 30 mins RT Before Use → 

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

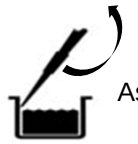
2. Avoid repeated cool-thaw of the components as there will be a loss of activity and this can affect the results.

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

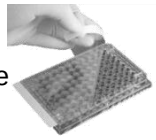
3.  Pipette **100 ul Standards and diluted Samples** into the respective wells.

4. Cover plate  and **incubate** for 

at 37^{0}C.

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

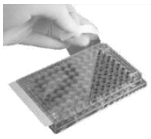
6.  Pipette **100 ul Cetuximab:HRP Conjugate** into each well.

7. Cover plate  and **incubate** for 

at 37^{0}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^{0}C.

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

12. 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	Results
1A 2A	0 Standard 0 Standard			
1B 2B	80 ng/ml 80 ng/ml			
1C 2C	160 ng/ml 160 ng/ml			
1D 2D	240 ng/ml 240 ng/ml			
1E 2E	320 ng/ml 320 ng/ml			
1F 2F	640 ng/ml 640 ng/ml			
1G 2G	*Sample*			
1H 2H	*Sample*			
3A 4A	*Sample*			
3B 4B	*Sample*			

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SYMBOLS KEY

MTP	Erbitux™ Cetuximab Coated Microtiter Plate (12 x 8 Wells)
STD	Human Anti-Cetuximab Standard
HRP CONJ	Cetuximab:HRP Conjugate
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