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Anti-Cetuximab ELISA

REF : KBI2018

Ver 1.0

RUO

Enzyme Immunoassay for the quantitative determination of Anti-Cetuximab in serum, plasma and cell culture supernatant

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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Introduction:

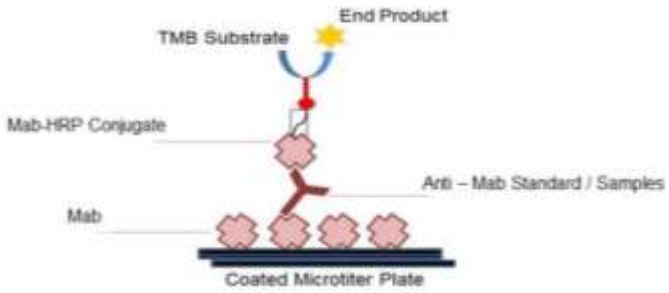
Cetuximab is an epidermal growth factor receptor (EGFR) inhibitor used for the treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer and head and neck cancer. Cetuximab is a chimeric (mouse/human) monoclonal antibody given by intravenous infusion that is distributed under the trade name Erbitux 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).Anti-Drug Antibodies (ADA) may induce unwanted side effects in biopharmaceuticals. Hence, ADA has been subjected to increase in scrutiny by the regulatory authorities using immunogenicity safety studies. ADA has been observed in pre-clinical and clinical studies, resulting in significant changes in toxicology, pharmacokinetics and efficacy. These effects result from the generation of drug-induced (neutralizing) autoantibodies against Cetuximab and can be responsible for allergic reaction, or even anaphylactic shock. This ELISA kit detects antibodies for Anti-Cetuximab and may be used for monitoring immunogenicity.

Intended Use:

The Anti-Cetuximab ELISA is used as an analytical tool for quantitative determination of Anti-Cetuximab in serum, plasma and cell culture supernatant.

Principle:

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



Materials Provided:

1. Cetuximab Coated Microtiter Plate (12x8 wells) – 1 no
2. Anti-Cetuximab Standard, (0.5 ml/vial) – 0, 10, 20, 40, 80, 160, 320 and 640 ng/ml
3. Cetuximab:HRP Conjugate – 12 ml
4. Assay Diluent – 6 ml
5. Sample Diluent – 50 ml
6. Wash Buffer (20X) – 25 ml
7. TMB Substrate – 12 ml
8. Stop Solution – 12 ml
9. Instruction Manual

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 25µl to 1000µl
3. Deionized (DI) water
4. Wash bottle or automated microplate washer

5. Semi-Log 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 In-Vitro Diagnostic 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 -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 1:10 to 1:100 (v/v), e.g. for 1:100 (5 µl sample + 495 µl 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 50 ml of 20X Wash Buffer in 950 ml of DI water.

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 Anti-Cetuximab. High Dose Hook Effect is due to excess of antibody for very high concentrations of Anti-Cetuximab 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 Anti-Cetuximab 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 Anti-Cetuximab.
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 Controls 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 out **50 µl** of **Assay Diluent** in each well.

3. Add **100 µl** of **Standards** or **Samples** into the respective wells.

4. Cover the plate and incubate for 60 minutes 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. Pipette without delay in the same order **100 µl** of **Cetuximab: HRP Conjugate** into each well.

7. Cover the plate and incubate for 60 minutes at 37°C.

8. 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.

9. Add **100 µl** of **TMB Substrate** in each well.

10. Incubate the plate at **37°C** for 15 minutes in dark. DO NOT SHAKE or else it may result in higher backgrounds and worse precision. Positive wells should turn bluish in color.

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

12. 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 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 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.

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 10 ng/ml

Linearity:

Standards provided in the kit will be used for measuring the linearity range of Anti-Cetuximab present in matrix.

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 (10ng/ml), medium (80ng/ml) and high (640ng/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%

Limitations of Method

Healthy individuals should be tested negative by the Anti-Cetuximab. Any clinical diagnosis should not be based on the results of in vitro diagnostic methods alone. Physicians are suggested to consider all clinical and laboratory findings possible to state a diagnosis.

Safety Precautions:

- **This kit is for in vitro 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:

Anti-cetuximab IgE ELISA for identification of patients at a high risk of cetuximab-induced anaphylaxis.Mariotte D, Dupont B, Gervais R, Galais MP, Laroche D, Tranchant A, Comby E, Bouhier-Leporrier K, Reimund JM, Le Mauff B….MAbs….2011…Taylor Francis

Standard chemotherapy with cetuximab for treatment of colorectal cancer… Li XX, Liang L, Huang LY, Cai SJ…World J Gastroenterol…2015…BPG

Risk factors associated with hypersensitivity reactions to cetuximab: anti-cetuximab IgE detection as screening test….Dupont B, Mariotte D, Clarisse B, Galais MP, Bouhier-Leporrier K, Grellard JM, Le Mauff B, Reimund JM, Gervais R….Future Oncol…2014 …Future Medicine

Cetuximab-mediated tumor regression depends on innate and adaptive immune responses…Yang X, Zhang X, Mortenson ED, Radkevich-Brown O, Wang Y, Fu YX…..Mol Ther….2013…Elsevier

A novel approach to predict cetuximab-induced hypersensitivity reaction: detection of drug-specific IgE on basophils…Iwamoto T, Okamoto A, Ishinaga H, Shimizu K, Gayle AA, Arai N, Takeuchi K, Okuda M….Cancer Med…2016….Wiley

SCHEMATIC ASSAY PROCEDURE

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

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

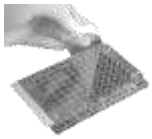
Thaw at Room Temperature (18-24ºC) → USE NOW

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 **50 µl Assay Diluent** into each well.

4.  Pipette **100 µl Standards** / **Samples** into the respective wells.

5. Cover plate  and incubate for 

at 37°C.

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

7.  Pipette **100 µl Cetuximab:HRP** into each well.

8. Cover plate  and incubate for 

at 37°C.

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

10.  Pipette **100 µl TMB Substrate** into each well.

11. Cover plate  and incubate for 

at 37°C.

12.  Pipette **100 µl Stop Solution** into each well.

13. 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 Anti-Cetuximab equivalent
1A 2A	zero std zero std			
1B 2B	10 ng/ml 10 ng/ml			
1C 2C	20 ng/ml 20 ng/ml			
1D 2D	40 ng/ml 40 ng/ml			
1E 2E	80 ng/ml 80 ng/ml			
1F 2F	160 ng/ml 160 ng/ml			
1G 2G	320 ng/ml 320 ng/ml			
1H 2H	640 ng/ml 640 ng/ml			
3A 4A	Sample			
3B 4B	Sample			

LIMITED WARRANTY

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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.

THANK YOU FOR USING KRISHGEN PRODUCT!

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