MATRIKS BIOTEK
innovation for health & wellness

"trace & catch"

Instruction for Use

Cetuximab (Erbitux®) ELISA

SHIKARI®
Q-CET

Enzyme immunoassay for the quantitative determination of cetuximab (Erbitux®) in serum and plasma

REF **TR-CETv1** 12 x8 2-8 C

Revision # 1.2 August 2017

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IVD

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	Free Cetuximab (Erbitux®) quantative analyse
Required Volume (µl)	10
Total Time (min)	70
Sample	Serum, plasma
Sample Number	96
Detection Limit (µg/mL)	10
Spike Recovery (%)	Between 85-115
Shelf Life (year)	1

Intended Use

Enzyme immunoassay for the quantitative determination of free cetuximab in serum and plasma. Matriks Biotek® cetuximab ELISA has been especially developed for the quantitative analysis of free cetuximab in serum and plasma samples.

Summary and Explanation

Epidermal growth factor receptor (EGFR)

Epidermal growth factor receptor (EGFR; HER1; ErbB1) is a transmembrane tyrosine kinase encoded by c-*erb*-B proto-oncogene, expressed in normal and malignant cells and stimulated by epidermal growth factor (EGF) or transforming growth factor-alpha (TGF-alpha) binding extracellular domain of the receptor, leading receptor to dimerize and activating intracellular kinase domain on each receptor, bringing about phosphorylation of tyrosine residues on each member of the receptor pair. Then, signaling complexes form in cytoplasm to activate gene transcription responding for such as cell proliferation. Termination of signaling occurs through internalization of receptor-ligand complex. Activation of EGFR results in perturbation of mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase, and AKT pathways triggering tumorigenic processes, such as increased proliferation, angiogenesis and metastasis, and prevents apoptosis. Breast, lung, colon, prostate, kidney, bladder, head and neck, and ovary cancers have been associated to EGFR overexpression which causes early disease progression, poor survival, and resistance to chemotherapy in many epithelial malignancies. Epidermal growth factor receptor/human epidermal growth factor receptor 1 (EGFR/HER1) and its ligand, transforming growth factor-alpha (TGF-alpha) were showed to involve in hepatocarcinogenesis. EGFR is overexpressed in hepatocellular carcinoma (HCC). To overcome the uncontrollable effect of EGFR triggering cancer development, monoclonal antibodies have been shown to be used as blockers *in vitro* and *in vivo*.

Cetuximab

Cetuximab (IMC-C225, Erbitux) is a chimeric monoclonal antibody of the immunoglobulin G1 (IgG1) and FDA-approved epidermal growth factor receptor (EGFR) inhibitor. It is a 152 kDa protein composed of four polypeptide chain. There are 32 cysteine residues forming accordingly 16 potential disulfide bonds. Preclinical studies have shown that cetuximab enhances the antitumour effects of chemotherapy (e.g. that of irinotecan in colorectal cancer) as well as radiotherapy (e.g. in squamous cell carcinoma of the head and neck) by inhibiting cell proliferation, angiogenesis and metastasis and by promoting apoptosis is used for the treatment of metastatic colorectal cancer, metastatic non-small cell lung cancer and head and neck cancer. Cetuximab also blocks growth factor-induced activation of the downstream mitogen-activated

protein kinase, inhibiting cell proliferation. It has been also illustrated that cetuximab increases the receptor internalization which is another mechanism to silence the receptor. Cetuximab arrests cell cycle at G1 gap phase by upregulating anti-proliferative $p27^{kip1}$, which functions via complex formation with Cdk2, and downregulating proliferating cell nuclear antigen (PCNA). It also decreases angiogenic factors, inhibits tumor-cell invasion and metastasis via downregulation of matrix metalloproteinases (MMPs) and VEGF, and promotes apoptosis by upregulating apoptotic protein, Bax, with the help of other chemotherapeutic agents. Cetuximab has been widely shown to display synergistic effect with other agents and/or radiotherapy.

Binding of antigen-binding fragment (Fab) of Cetuximab, which displays higher affinity comparing to ligands of EGFR, takes place via domain III of extracellular EGFR, preventing the receptor from conformational change to be dimerized and blocking EGFR signaling through inhibition of EGF and TGF-alpha-stimulated phosphorylation of the receptor.

Pharmacokinetics and Pharmacodynamics

In a study conducted by Fracasso et al., patients with colorectal, breast, and head and neck carcinomas were administered with one of different dosages of cetuximab (50, 100, 250, 400 and 500 mg/m^2). For each concentration, cetuximab serum concentration was showed to reach maximum at 3 h, and decrease slowly. Serum concentration decreased to baseline at 96 h and 168 h for dosages 50 and 100 mg/m^2, respectively. Mean maximum observed concentrations (C_{max}) increased in a dose dependent manner (from 22.8 ug/ml to 245.6 ug/ml).

It was indistinguishable for 400 mg/m^2 (C_{max}=228.9 ug/ml) and 500 mg/m^2 (C_{max}=245.6 ug/ml). The mean total body clearance based on body surface area for cetuximab was similar following doses of >100 mg/m^2 (range, 34.4-19.3 L/h/m^2) but greater in the 50 mg/m^2 dose group (65.9 L/h/m^2). Biopsy results showed that maximal cytoplasmic EGFR downregulation after treatment was seen in 8 h with 400 mg/m^2 dosage.

After 250 mg/m^2 weekly cetuximab administration, the average trough level of patients with both partial responses (PRs) and stable disease (SD) was 60,742 ng/ml (~400 nmol/l) compared with those patients with progressive disease (PD; 33,208 ng/ml). In another study, cetuximab was infused as loading dose of 400 mg/m^2 followed by weekly infusions of 250 mg/m^2 in colorectal cancer patients. Median residual concentrations were 41 and 54 mg/L on days 14 and 28, respectively. It was determined that initial serum albumin concentration was significantly related to first-order elimination clearance of cetuximab. Central volume of distribution was 2.96 L (4%), peripheral volume of distribution was 4.65 L (6%), elimination clearance was 0.479 L/d (4%) and distribution clearance was 0.836 L/d (8%).

Test Principle

Solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. Standards and samples (serum or plasma) are incubated in the microtitre plate coated with the reactant for cetuximab (Erbitux®). After incubation, the wells are washed. A horse radish peroxidase (HRP) conjugated probe is added and binds to cetuximab (Erbitux®) captured by the reactant on the surface of the wells. Following incubation wells are washed and the bound enzymatic activity is detected by addition of chromogen-substrate. The colour developed is proportional to the amount of cetuximab (Erbitux®) in the sample or standard. Results of samples can be determined directly using the standard curve.

Warnings and Precautions

1. For professional use only.
2. Before starting the assay, read the instructions completely and carefully. Use the valid version of the package insert provided with the kit. Be sure that everything is understood. For further information (clinical background, test performance, automation protocols, alternative applications, literature, etc.) please refer to the local distributor.
3. In case of severe damage of the kit package please contact Matriksbiotek or your supplier in written form, latest one week after receiving the kit. Do not use damaged components in test runs, but keep safe for complaint related issues.
4. Obey lot number and expiry date. Do not mix reagents of different lots. Do not use expired reagents.
5. Follow good laboratory practice and safety guidelines. Wear lab coats, disposable latex gloves and protective glasses where necessary.
6. Reagents of this kit containing hazardous material may cause eye and skin irritations. See MATERIALS SUPPLIED and labels for details.
7. Chemicals and prepared or used reagents have to be treated as hazardous waste according the national biohazard safety guidelines or regulations.

8. Avoid contact with Stop solution. It may cause skin irritations and burns.

9. Some reagents contain sodium azide (NaN3) as preservatives. In case of contact with eyes or skin, flush immediately with water. NaN3 may react with lead and copper plumbing to form explosive metal azides. When disposing reagents, flush with large volume of water to avoid azide build-up.

10. All reagents of this test kit containing human serum or plasma have been tested and were found negative for HIV I/II, HBsAg and HCV by FDA approved procedures. However, a presence of these or other infectious agents cannot be excluded absolutely and therefore reagents should be treated as potential biohazards in use and for disposal.

Storage and Stability

The kit is shipped at ambient temperature and should be stored at 2-8°C. Keep away from heat or direct sun light. The storage and stability of specimen and prepared reagents is stated in the corresponding chapters. The strips of microtiter plate is stable up to the expiry date of the kit in the broken, but tightly closed bag when stored at 2–8°C.

Specimen Collection and Storage

Serum, Plasma (EDTA, Heparin)*

The usual precautions for venipuncture should be observed. It is important to preserve the chemical integrity of a blood specimen from the moment it is collected until it is assayed. Do not use grossly hemolytic, icteric or grossly lipemic specimens. Samples appearing turbid should be centrifuged before testing to remove any particulate material.

Storage:	2-8°C	-20°C	Keep away from heat or direct sun light
Stability:	7 d	6 mon	Avoid repeated freeze-thaw cycles

*. Cetuximab (Erbitux®) infusion camouflages/masks the presence of antibody to cetuximab in serum/plasma samples. Therefore, blood sampling time is critical for detection of cetuximab. Matriks Biotek® Laboratories propose to obtain blood sample just before the infusion of cetuximab (Erbitux®) or at least 2 weeks after the infusion of cetuximab (Erbitux®).

Materials Supplied

1 x 12 x 8	MTP	**Microtiter Plate** Break apart strips. Microtiter plate with 12 rows each of 8 wells coated with reactant.
7 x 0.3 mL	STND A-E HIGH CNTRL LOW CNTRL	**Cetuximab Standarts A-E (100x), High Level Control (100x), Low Level Control (100x)** 300; 100; 30; 10; 0 microgram/mL Used for construction of the standard curve. Contains cetuximab (Erbitux®), human serum, stabilizer and <0.1 % NaN_3
2 x 50 mL	ASSAY BUF	**Assay Buffer** Blue colored. Ready to use. Contains proteins, and <0.1% NaN3
1 x 12 mL	HRP CONJ	**Horse Radish Peroxidase Conjugate** Red colored. Ready to use. Contains HRP conjugate, stabilizer and preservatives.
1 x 12 mL	TMB SUBS	**TMB Substrate Solution** Ready to use. Contains TMB
1 x 12 mL	TMB STOP	**TMB Stop Solution** Ready to use. 1N HCl.
1 x 50 mL	WASHBUF CONC	**Wash Buffer, Concentrate (20x)** Contains Buffer with Tween 20.
2 x 1	ADH FILM	**Adhesive Film** For covering of Microtiter Plate during incubation.
2x 1	SLGP	**Semi-Log Graph Paper** For constructing standard curve and calculation of results.

Materials Required but not Supplied

1. Micropipettes (< 3% CV) and tips to deliver 5-1000 µL.
2. Calibrated measures.
3. Tubes (1 mL) for sample dilution.
4. Wash bottle, automated or semi-automated microtiter plate washing system.
5. Microtiter plate reader capable of reading absorbance at 450/650 nm.
6. Bidistilled or deionised water, paper towels, pipette tips and timer.

Procedure Notes

1. Any improper handling of samples or modification of the test procedure may influence the results. The indicated pipetting volumes, incubation times, temperatures and pretreatment steps have to be performed strictly according to the instructions. Use calibrated pipettes and devices only.
2. Once the test has been started, all steps should be completed without interruption. Make sure that required reagents, materials and devices are prepared ready at the appropriate time. Allow all reagents and specimens to reach room temperature (18-25 °C) and gently swirl each vial of liquid reagent and sample before use. Mix reagents without foaming.
3. Avoid contamination of reagents, pipettes and wells/tubes. Use new disposable plastic pipette tips for each reagent, standard or specimen. Do not interchange caps. Always cap not used vials. Do not reuse wells/tubes or reagents.
4. Use a pipetting scheme to verify an appropriate plate layout.
5. Incubation time affects results. All wells should be handled in the same order and time sequences. It is recommended to use an 8-channel Micropipettor for pipetting of solutions in all wells.
6. Microplate washing is important. Improperly washed wells will give erroneous results. It is recommended to use a multichannel pipette or an automatic microplate washing system. Do not allow the wells to dry between incubations. Do not scratch coated wells during rinsing and aspiration. Rinse and fill all reagents with care. While rinsing, check that all wells are filled precisely with Wash Buffer, and that there are no residues in the wells.
7. Humidity affects the coated wells/tubes. Do not open the pouch until it reaches room temperature. Unused wells/tubes should be returned immediately to the resealed pouch including the desiccant.

1. Preparation of Component

Dilute/ disolve	Component	with	Diluent	Relation	Remarks	Storage	Stability
10 mL	Wash Buffer*	Up to 200 mL	Bidist. water	1:20	Warm up at 37°C to dissolve crystals. Mix vigorously.	2-8 °C	2 w

*. Prepare Wash Buffer before starting assay procedure.

2. Dilution of Standarts and Samples (serum/plasma)

Standart/ Sample	To be diluted	With	Remarks
10 µL	1:100	Assay Buffer	For dilution at 1:100; 10µl Standart or Sample +990µl Assay Buffer

Patient samples with a concentration of cetuximab above the measuring range are to be rated as > "Highest Standard (Standard A)". The result must not be extrapolated. The patient sample in question should be further diluted with Assay Buffer and retested.

Test Procedure

1	Dilute each of the standards and samples (serum/plasma) at 1:100 using Assay Buffer as described in "Dilution of Standards and Samples (serum/plasma)" section
2	Pipette 100µl of Assay Buffer non-exceptionally into each of the wells to be used
3	Pipette 10 µL of Diluted Standards, Diluted High Level Control, Diluted Low Level Control and Diluted Samples into the respective wells of microtiter plate. **Wells** A1: Diluted Standard A B1: Diluted Standard B C1: Diluted Standard C D1: Diluted Standard D E1: Diluted Standard E F1: Diluted High Level Control G1: Diluted Low Level Control H1 and on: Diluted Sample (Serum/Plasma)
4	Cover the plate with adhesive film. Incubate 30 min at room temperature (18-25°C).
5	Remove adhesive film. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
6	Pipette 100 µL of ready-to use HRP Conjugate into each well.
7	Cover the plate with adhesive film. Incubate 30 min at room temperature (18- 25°C).
8	Remove adhesive film. Discard incubation solution. Wash plate 3 times each with 300 µL of diluted Wash Buffer. Remove excess solution by tapping the inverted plate on a paper towel.
9	Pipette 100 µL of TMB Substrate Solution into each well.
10	Incubate 10 min (without adhesive foil.) at room temperature (18-25°C) in the dark.
11	Stop the substrate reaction by adding 100 µL of Stop Solution into each well. Briefly mix contents by gently shaking the plate. Color changes from blue to yellow
12	Measure optical density with a photometer at 450/650 nm within 30 min after pipetting of the Stop Solution.

Quality Control

The test results are only valid only if the test has been performed following the instructions. Moreover the user must strictly adhere to the rules of GLP (Good Laboratory Practice) or other applicable standards/laws. All standards must be found within the acceptable ranges as stated above and/or label. If the criteria are not met, the run is not valid and should be repeated. In case of any deviation the following technical issues should be proven: Expiration dates of (prepared) reagents, storage conditions, pipettes, devices, incubation conditions and washing methods.

Calculation & Interpretation of Results

1. Using the diluted standards (3000; 1000; 300; 100; 0 ng/mL) disregarding zero standard, construct a standard curve by plotting the OD450/650 nm for each of 4 standards on the vertical (Y-axis) axis versus the corresponding cetuximab concentration on the horizontal (X-axis) axis, thus creating a standard curve by 4 points obtained.

2. The concentration of the samples can be read directly from this standard curve. Using the absorbance value for each sample, determine the corresponding concentration of cetuximab from the standard curve. Find the absorbance value on the Y-axis and extend a horizontal line to the curve. At the point of intersection, extend a vertical line to the X-axis and read the cetuximab concentration for the unknown sample.

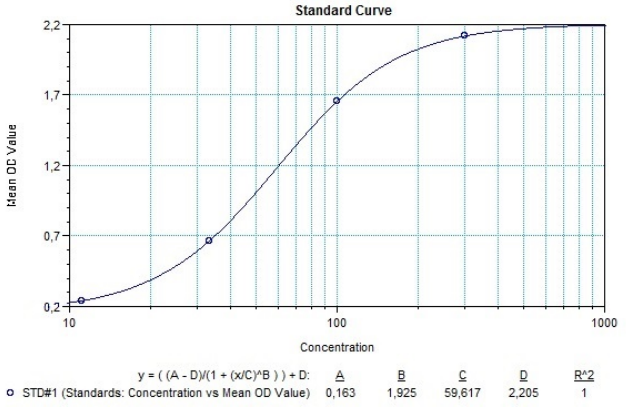
3. If computer data regation is going to be used, we recommend primarily "4 Parameter Logistic (4PL)" or secondly the "point-to-point calculation".

4. To obtain the exact values of the samples, the concentration determined from the standard-curve must be multiplied by the dilution factor (100x). Any sample reading greater than the highest standard should be further diluted appropriately with Assay Buffer and retested. Therefore, if the pre-diluted samples have been further diluted, the concentration determind from the standard curve must be multiplied by the further dilution factor.

   E.g.; *If the pre-diluted sample further diluted in a ratio of 1:5 then results should be multiplied by 500.*

5. Automated method: Computer programs can also generally give a good fit.

Typical Calibration Curve
(Example. Do not use for calculation!)



Standart	Concentration (ng/mL)	Mean OD450/650
A	3000	2,118
B	1000	1,654
C	300	0,666
D	100	0,240
E	0	0,055

Assay Characteristics

1. **Specificity:** There is no cross reaction with native serum immunoglobulins.

2. **Sensitivity:** The lowest detectable level that can be distinguished from the zero standard is 100 ng/mL.

3. **Precision Of Kit:**
   Intra-assay CV: <15% for cetuximab range 100-3000 ng/mL.
   Inter-assay CV: <15% for cetuximab range 100-3000 ng/mL.

4. **Recovery:** Recovery rate was found to be between 85-115% with normal human serum samples with known concentrations.

Automation

Experiments have shown that the *Matriks Biotek®* SHIKARI® Cetuximab ELISA is also suitable to run on an automated ELISA processor.

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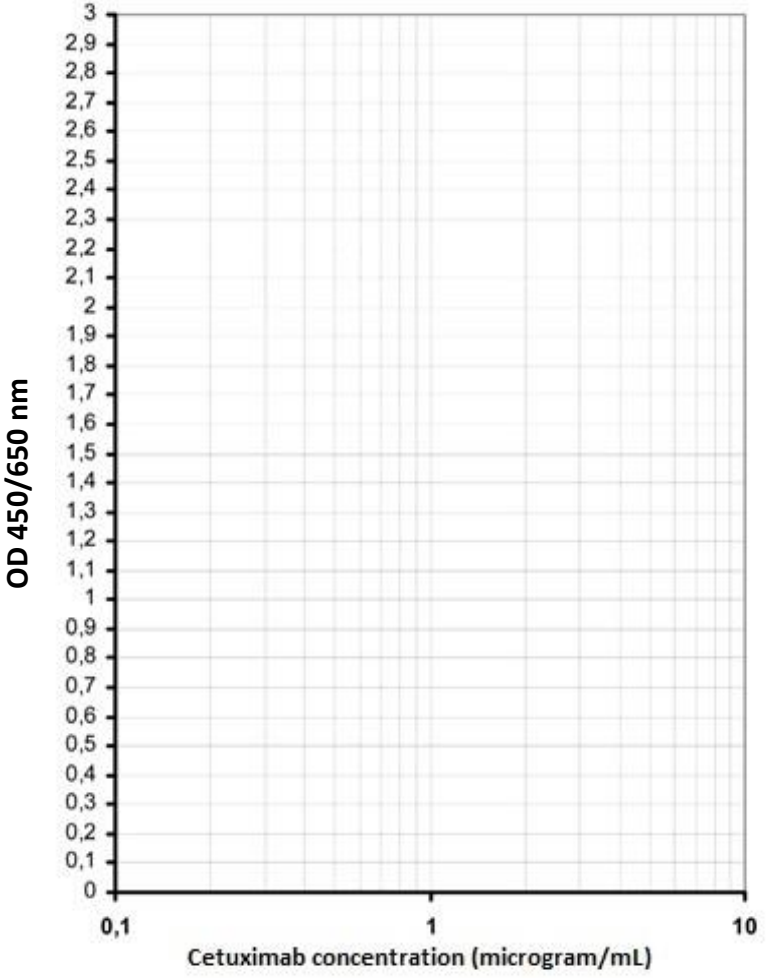
OD 450/650 nm
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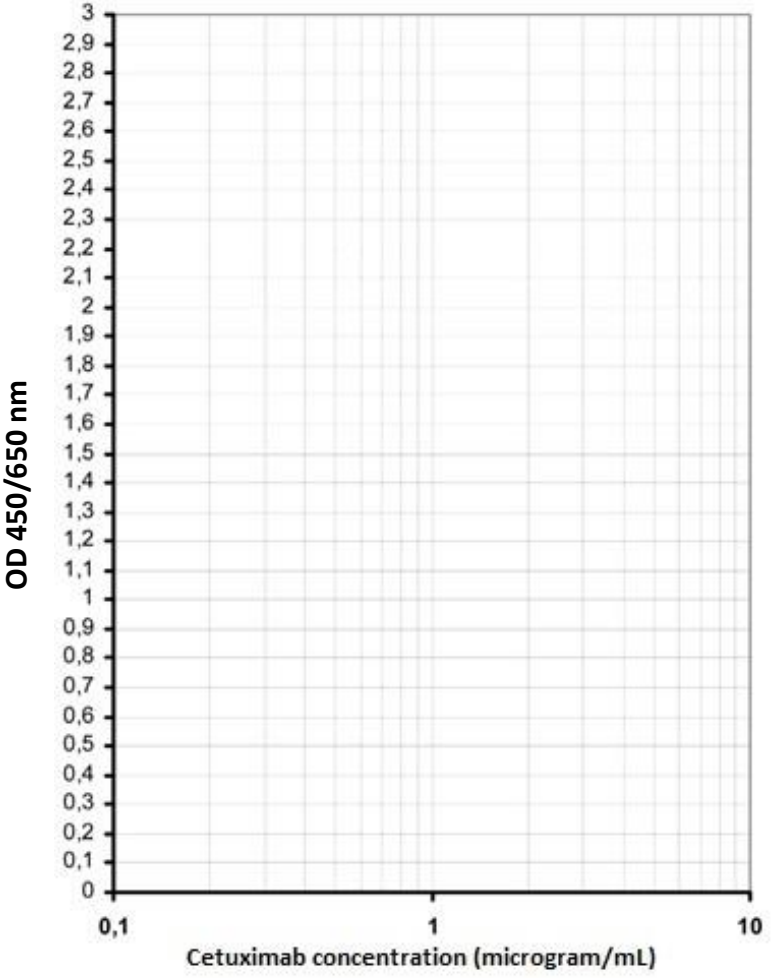
10
Cetuximab concentration (microgram/mL)


OD 450/650 nm
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Cetuximab concentration (microgram/mL)