Epitope Diagnostics,Inc.

For Reference Use Only

EDI™ Intact Brentuximab Vedotin ELISA Kit

Enzyme Linked ImmunoSorbent Assay (ELISA) for the Quantitative Measurement of Brentuximab Vedotin Level in Serum or Plasma

REF **KT-705** EU: IVD CE Σ 96 +8 +2 °C

I. INTENDED USE

This highly sensitive "sandwich" test kit is intended for use in the quantitative determination of intact brentuximab vedotin level in serum or plasma. It is useful for clinical monitoring of therapeutic drug (brentuximab vedotin) concentration for precision treatment.

II. ASSAY PRINCIPLE

This ELISA kit is designed, developed and produced for the quantitative measurement of brentuximab vedotin in serum or plasma. The assay utilizes a sandwich immunoassay technique with an antibody that binds to MMAE. Briefly, a mouse monoclonal antibody specific to MMAE is coated onto a microtiter plate. In the assay system, the assay calibrators, controls and test specimen are added to this microtiter plate. During the first incubation period, the anti-MMAE monoclonal antibody captures the MMAE-Antibody Conjugate of calibrators, controls, and test samples. Unbound proteins are washed away with a wash step. A HRP (horseradish peroxidase) conjugate anti-human IgG tracer antibody is added to each well of the microtiter plate. After the second incubation, a "sandwich" immunocomplex of "Anti-MMAE antibody – MMAE Antibody Conjugate – HRP-conjugated anti-human IgG antibody" is formed and attached to the wall of the plate. The unbound HRP-conjugated antibody is removed in a subsequent washing step. For the detection of this immunocomplex, each well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immunocomplex bound to MMAE Antibody Conjugate on the wall of the microtiter well is directly proportional to the amount of MMAE Antibody Conjugate level in the sample.

III. REAGENTS: Preparation and Storage

This test kit must be stored at 2 – 8°C upon receipt. For the expiration date of the kit refer to the label on the kit box. All components are stable until this expiration date.

Prior to use allow all reagents to come to room temperature. Regents from different kit lot numbers should not be combined or interchanged.

1. **Anti-MMAE Antibody Coated Microplate (Cat. No. 30793)**
   One microplate with twelve by eight strips (96 wells total) coated with monoclonal MMAE antibody. The plate is framed and sealed in a foil zipper bag with a desiccant. This reagent should be stored at 2 – 8 °C and is stable until the expiration date on the kit box.

2. **MMAE Tracer Antibody (Cat. No. 30753)**
   One vial containing **12 mL** of ready to use MMAE Tracer Antibody in a stabilized protein matrix. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

3. **ELISA Wash Concentrate (Cat. No. 10010)**
   One bottle containing **30 mL** of 30-fold concentrate. Before use the contents must be diluted with **870 mL** of demineralized water and mixed well. Upon dilution, this yields a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non-mercury preservative. The diluted wash solution may be stored at room temperature and is stable until the expiration date on the kit box.

4. **ELISA HRP Substrate (Cat. No. 10020)**
   One bottle containing **15 mL** of tetramethylbenzidine (TMB) with hydrogen peroxide. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

5. **ELISA Stop Solution (Cat. No. 10030)**
   One bottle containing **15 mL** of stop solution. This reagent may be stored at 2 – 8°C or room temperature and is stable until the expiration date on the kit box.

6. **Assay Buffer (Cat. No. 30779)**
   One bottle containing 12 mL ready-to-use buffer. It should be used according to the assay procedures. This reagent should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

7. **Antibody Conjugated Calibrator Zero (Cat. No.30759)**
   One vial containing **30 mL** calibrator zero (30759). This reagent is used for diluting the calibrator stock to make assay calibrators, as well as for diluting test samples. This reagent should be stored at 2-8ºC and is stable until the expiration date on the kit box.

8. **Antibody-MMAE Conjugated Calibrator Stock (Cat. No. 30794*)***
   One vial containing lyophilized antibody MMAE conjugated calibrator stock in a serum based matrix with non-azide preservative. **Refer to the vial for exact concentration of the calibrator.** This calibrator should be stored at 2 – 8°C and is stable until the expiration date on the kit box.

IV. SAFETY PRECAUTIONS

The reagents must be used in professional laboratory. Source material for reagents containing bovine serum was derived in the contiguous 48 United States. It was obtained only from healthy donor animals maintained under veterinary supervision and found free of contagious diseases. Wear gloves while performing this assay and handle these reagents as if they are potentially infectious. Avoid contact with reagents containing TMB, hydrogen peroxide, or sulfuric acid. TMB may cause irritation to skin and mucous membranes and cause an allergic skin reaction. TMB is a suspected carcinogen. Sulfuric acid may cause severe irritation on contact with skin. Do not get in eyes, on skin, or on clothing. Do not ingest or inhale fumes. On contact, flush with copious amounts of water for at least 15 minutes. Use Good Laboratory Practices.

V. MATERIALS REQUIRED BUT NOT PROVIDED

1. Precision single channel pipettes capable of delivering 25 µL, 50 µL, 100 µL, etc.
2. Disposable pipette tips suitable for above volume dispensing.
3. Aluminum foil.
4. Deionized or distilled water.
5. Plastic microtiter well cover or polyethylene film.
6. ELISA multichannel wash bottle or automatic (semi-automatic) washing system.
7. Spectrophotometric microplate reader capable of reading absorbance at 450/650 or 450/620 nm.
8.

VI. SPECIMEN COLLECTION

Serum or EDTA-plasma samples are suitable specimens for MMAE-ADC measurement. Only **10 µL** of samples is required for a duplicate determination of MMAE-ADC with this test kit. No special preparation of individual is necessary prior to specimen collection. Samples should be collected by standard technologies of clinical laboratory practice and recommended by manufacturer of sample collection tube. It is extremely important to carefully separate the plasma from blood cells to avoid hemolyzation, etc. Samples should be transferred to a clean test tube right after centrifugation and should be stored at 2 – 8ºC if the assay is to be performed within 72 hours. Otherwise, patient samples should be stored at –20ºC or below until measurement. Avoid more than three times freeze-thaw cycles of specimen. Do not use hemolyzed, hyperlipermic, heat-treated or any contaminated specimens.

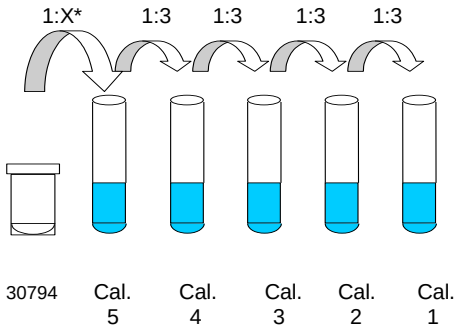
VII. ASSAY PROCEDURE

1. Reagent Preparation

(1) Prior to use allow all reagents to come to room temperature. Reagents from different kit lot numbers should not be combined or interchanged.

(2) ELISA Wash Concentrate (Cat. 10010) must be diluted to working solution prior to use. Please see REAGENTS section for details.

(3) Using EDI Calibrator Stock (Cat 30794):
Reconstitute calibration stock 30794 with **0.5 mL** DI-water. Dilute the reconstituted calibration stock (30794) 1:X* using the zero calibrator (30759) to obtain a level five calibrator at 390 ng/mL. Further create calibrator level four to one by 1:3 serial dilutions to obtain these calibrators with concentrations of 130 ng/mL, 43.3 ng/mL,14.4 ng/mL, 4.8 ng/mL. Assay calibrators should be used within 2 hours and should be stored below -20°C. Do not exceed 3 freeze-thaw cycles.



X* = the concentration of 30794 / 390

Example of making Calibrators 1-5:

Calibrator	Calibrator Volume	Dilution Factor	Volume of Cal 0
Stock 1000 ng/mL	*(refer to the label for exact stock concentration, Cat#30794)*	1:2.56 (1:X*)	-
Cal 5 390 ng/mL	0.39 mL of stock	1:3	0.610 mL
Cal 4 130 ng/mL	0.5 mL of Cal 5	1:3	1 mL
Cal 3 43.3 ng/mL	0.5 mL of Cal 4	1:3	1 mL
Cal 2 14.4 ng/mL	0.5 mL of Cal 3	1:3	1 mL
Cal 1 4.8 ng/mL	0.5 mL of Cal 2	-	1 mL
Cal 0 0 ng/mL	*Cat# 30759*	-	-

The validation data of this test was generated by using EDI Antibody-MMAE Conjugated Calibrator Stock. To order this calibrator stock, please order Ab-MMAE Conjugated Stock (Cat. No. 30794).

(4) When using own brentuximab vedotin or MMAE-ADC calibrator stock, the user may follow step 3 (*Using EDI Calibrator Stock*) as a reference. Every ADC is created with different DARs and conjugation methods. It is recommended to make in-house calibration curve.

(5) Dilute each unknown sample **1:100** using **Antibody Conjugated Calibrator Zero (Cat# 30759).**

(6) Place a sufficient number of Anti-MMAE antibody coated microwell strips in a holder to determine calibrators and diluted unknown samples in duplicate.

2. Assay Procedure:

Patient serum or plasma samples may have to be diluted with zero standard matrix before testing.

(1) Add **25 µL** of calibrators and **diluted 1:100** test samples into the designated microwells. Tap the plate gently.

(2) Immediately add **100 µL** of Assay Buffer (Cat# 30799)

(3) Seal the plate wells securely, cover with foil or similar material to protect from light, and rotate on an ELISA plate shaker (small orbit radius) for **1 hour** at 400 to 450 rpm.

(4) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.

(5) Add **100 µL** of MMAE Tracer Antibody (Cat# 30753) to each well. Tap the plate gently.

(6) Seal the plate wells securely, cover with foil or similar material to protect from light, and rotate on an ELISA plate shaker (small orbit radius) for **30 minutes** at 400 to 450 rpm.

(7) Wash each well 5 times by dispensing 350 µL of working wash solution into each well and then completely aspirating the contents. Alternatively, an automated microplate washer can be used.

(8) Add 1**00 µL** of ELISA HRP Substrate into each of the wells.

(9) Cover the plate with aluminum foil or similar material to avoid exposure to light. Incubate plate static, at room temperature for **20 minutes**.

(10) Immediately add **100 µL** of ELISA Stop Solution into each of the wells. Mix gently.

(11) Read the absorbance at 450 nm with reference filter at 620 nm.

VIII. PROCEDURAL NOTES

1. It is recommended that all standards and unknown samples be assayed in duplicate. The average absorbance reading of each duplicate should be used for data reduction and the calculation of results. It is recommended to add external controls to each assay.
2. Keep light sensitive reagents in the original amber bottles.

3. Store any unused antibody coated strips in the foil zipper bag with desiccant to protect from moisture.
4. Careful technique and use of properly calibrated pipetting devices are necessary to ensure reproducibility of the test.
5. Incubation times or temperatures other than those stated in this insert may affect the results.
6. An orbital mixer with a larger orbit radius (e.g. > 1 cm) may be used at speeds of 150 to 200 rpm.
7. Avoid air bubbles in the microwell as this could result in lower binding efficiency and higher CV% of duplicate reading.
8. All reagents should be mixed gently and thoroughly prior to use. Avoid foaming.
9. If adapting this assay to automated ELISA system such as DS-2, DSX or Trituras, a procedural validation is necessary if there is any modification of the assay procedure.

IX. INTERPRETATION OF RESULTS

It is recommended to use a point to point standard curve fitting.

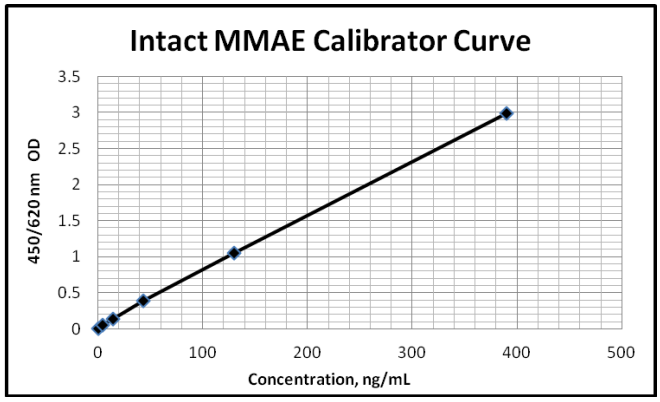
1. Calculate the average absorbance for each pair of duplicate test results.
2. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration. Appropriate computer assisted data reduction programs should be used for the calculation of results.

The antibody-MMAE conjugate concentrations for the test samples are read directly from the standard curve using their respective corrected absorbance.

X. EXAMPLE DATA AND STANDARD CURVE

A typical absorbance data and the resulting standard curve from this MMAE ADC ELISA are represented. **This curve should not be used in lieu of standard curve generated with each assay.**

Well I.D.	OD 450/620 nm Absorbance		
	Readings	Average	Corrected
0 ng/mL	0.009 0.010	0.010	0.000
4.8 ng/mL	0.052 0.058	0.055	0.045
14.4 ng/mL	0.147 0.136	0.141	0.131
43.3 ng/mL	0.373 0.418	0.395	0.385
130.0 ng/mL	1.114 0.991	1.052	1.042
390.0 ng/mL	2.901 3.080	2.991	2.981



XI. LIMITATION OF THE PROCEDURE

1. This assay requires serum or plasma sample for testing.
2. For sample values greater than 390 ng/mL, it is recommended to re-assay samples with further dilution with calibrator zero.
3. The kit standards are based on MMAE conjugated antibody or ADC concentration. It is not based on free MMAE concentration. The MMAE-ADC in different linker and DAR may give different curve shift
4. Brentuximab Vendotin dosage must be established for desired patient concentration. Proper dosage is ultimately left to the discretion of the doctor

XII. QUALITY CONTROL

To assure the validity of the results each assay should include adequate controls.

XIII. PERFORMANCE CHARACTERISTICS

Sensitivity

The sensitivity (lowest limit of detection, LLOD) of this MMAE-ADC ELISA as determined by the corresponding OD value of 2-fold standard deviation above the mean on 8 duplicate determination of zero standard is 0.112 ng/mL. Considering the 1:100 pre-dilution factor of serum or plasma samples, the actual test sensitivity for test sample is about 1.12 ng/ml.

The measurement rage for this MMAE-ADC ELISA is 1.12 ng/ml to 390.0 ng/ml.

Specificity

This brentuximab vedotin ELISA doesn't show any cross reactivity to DM1-ADC. This assay does cross react to MMAF-ADC. This test detect only the intact brentuximab vedotin, but not the free MMAE or the free monoclonal antibody.

High Dose "hook" effect

This assay has showed that it didn't have any high dose "hook" effect for MMAE ADC levels up to 1,000,000 ng/mL.

Precision

The intra-assay precision was validated by measuring three spiked samples with 16 replicate determinations.

Sample #	Mean Value (ng/mL)	CV (%)
1	34.28	2.7
2	11.33	4.7
3	19.9	7.9

The inter-assay precision was validated by measuring two control levels in duplicate in 8 individual assays.

Sample #	Mean Value (ng/mL)	CV (%)
1	15.71	5.3
2	126.05	4.8

Linearity

Two 1:100 diluted samples were spiked and diluted with standard zero and tested. The results of MMAE ADC dilution recovery value are as follows:

DILUTION	OBSERVED VALUE (ng/mL)	RECOVERY %
Sample A 1:100	156.0	-
1:2	73.1	93.7
1:4	40.1	102.9
1:8	21.2	108.6
Sample B 1:100	265.3	-

1:2	118.3	89.2
1:4	54.7	82.5
1:8	32.3	97.4

Spike Recovery

Two samples are equal volume mixed with standard concentrations 25, 62.4, and 156 ng/mL and tested. The results are as follows:

Spiked Sample	OBSERVED VALUE (ng/mL)	RECOVERY %
Sample A 1:100	40.1	-
25 ng/mL	34.5	105.8
62.4 ng/mL	48.4	94.4
156 ng/mL	98.9	100.9
Sample B 1:100	42.4	-
25 ng/mL	35.6	105.6
62.4 ng/mL	49.4	94.2
156 ng/mL	101.3	102.2

XIV. WARRANTY

This product is warranted to perform as described in its labeling and literature when used in accordance with all instructions. Epitope Diagnostics, Inc. DISCLAIMS ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE, and in no event shall Epitope Diagnostics, Inc. be liable for consequential damages. Replacement of the product or refund of the purchase price is the exclusive remedy for the purchaser. This warranty gives you specific legal rights and you may have other rights, which vary from state to state.

XV. REFERENCES

1. Sandhya Girish, et al. Clinical pharmacology of trastuzumab emtansine (T-DM1): an antibody–drug conjugate in development for the treatment of HER2-positive cancer. Cancer Chemother Pharmacol (2012) 69:1229–1240

TECHNICAL ASSISTANCE AND CUSTOMER SERVICE

For technical assistance or place an order, please contact Epitope Diagnostics, Inc. at (858) 693-7877 or fax to (858) 693-7678.
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MDSS GmbH
Schiffgraben 41
30175 Hannover, Germany

Manufacturer	No. of tests
REF Catalog Number	Keep away from heat and direct sun light
CONC Concentrate	Store at
IVD In Vitro Diagnostic Device	Use by
Read instructions before use	LOT Lot No.
EC REP Authorized Representative In Europe	

Brentuximab Vedotin ELISA: Condensed Assay Protocol

1. **25 µl standards and Diluted unknown samples**

   \+

   100 µl Assay Buffer

   ↓ *Incubate @ RT for 60 min on ELISA plate shaker Wash 5x*

2. **100 µl Tracer Antibody**

   ↓ *Incubate @ RT for 30 min on ELISA plate shaker Wash 5 x*

3. **100 µl TMB Substrate**

   ↓ *Incubate @ RT for 20 min static*

4. **100 µl Stop Solution**

   ↓ *Immediately*

5. **Read absorbance at 450 nm with reference filter at 620 nm** *within 10 minutes*