Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
fax 02-92157285
info@listarfish.it
www.listarfish.it

EDI™ Intact MMAF ADC ELISA Kit

Enzyme Linked ImmunoSorbent Assay (ELISA) for the Quantitative Measurement of Antibody MMAF Conjugate Level in Blood

REF **KTR-783** Σ 96 +2 +8 °C

For Research Use Only **Not for Use in Diagnostic Procedures**

I. INTENDED USE

This highly sensitive "sandwich" test kit is intended for use in the quantitative determination of antibody MMAF conjugate level in serum or plasma of human, mouse, rat, primate, etc. in less than 2 hours. It is useful for pre-clinical and clinical pharmacology study of MMAF Antibody Drug Conjugate (ADC).

II. ASSAY PRINCIPLE

This ELISA kit is designed, developed and produced for the quantitative measurement of antibody MMAF conjugate in serum or plasma. The assay utilizes the sandwich immunoassay technique with an antibody that binds to MMAF. Briefly, a mouse monoclonal antibody specific to MMAF 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-MMAF monoclonal antibody captures the MMAF-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-MMAF antibody – MMAF 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 MMAF Antibody Conjugate on the wall of the microtiter well is directly proportional to the amount of MMAF 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-MMAF Antibody Coated Microplate (Cat. No. 30796)**
One microplate with twelve by eight strips (96 wells total) coated with monoclonal MMAF 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. **MMAF Tracer Antibody (Cat. No. 30754)**
One vial containing **0.6 mL** of ready to use MMAF Tracer Antibody in a stabilized protein matrix. This reagent should be diluted before use (see reagent preparation for details) and stored at 2 – 8°C and is stable until the expiration date on the kit box.

3. **Tracer Antibody Diluent (Cat. No. 30710)**
One bottle containing **12 mL** ready-to-use buffer. It should be used only for tracer antibody dilution 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.

4. **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.

5. **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.

6. **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.

7. **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.

8. **Antibody Conjugated Calibrator Zero (Cat. No.30759)**
One vial containing **30 mL** calibrator zero (30759). This reagent is used for diluting the calibrator 6 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.

9. **Antibody-MMAF Conjugated Calibrator stock (Cat. No. 30797)- *Not provided in the kit, (may order separately)***
One vial containing lyophilized antibody MMAF 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.

VI. SPECIMEN COLLECTION

Serum or EDTA-plasma samples are suitable specimens for MMAF-ADC measurement. Only **10 µL** of samples is required for a duplicate determination of MMAF-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.

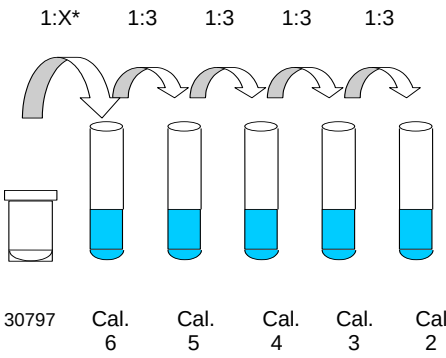
VII. ASSAY PROCEDURE

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 MMAF Calibrator Stock (Cat# 30797): Reconstitute calibration stock 30797 with **0.5 mL** DI-water. Dilute the reconstituted calibration stock (30797) 1:X* using the zero calibrator (30759) to obtain a level six calibrator at 270 ng/mL. Further create calibrator level five to two by 1:3 serial dilutions to obtain these calibrators with concentrations of 90 ng/mL, 30 ng/mL, 10 ng/mL, 3.3 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 30797 / 270

The validation data of this test was generated by using <u>EDI Antibody-MMAF Conjugated Calibrator stock (Cat. No. 30797)</u>! To order this calibrator stock, please order Ab-MMAF Conjugated stock (Cat. No.30797).

(4) Each unknown sample needs to be diluted **1:100** using **Antibody Conjugated Calibrator Zero.**

(5) Prepare MMAF Tracer Antibody working solution by **1:21** fold dilution of the MMAF Tracer Antibody (Cat. 30752) by adding the Tracer Antibody into the Tracer Antibody Diluent (Cat. 30710). Following is a table that outlines the relationship of strips used and antibody mixture prepared. ***NOTE:*** *the Tracer Antibody should be prepared just prior to use.*

Dilution Scheme	Tracer Antibody Diluent	Tracer Antibody
1	1 mL	50 µL
2	2 mL	100 µL
3	3 mL	150 µL
4	4 mL	200 µL
5	5 mL	250 µL
6	6 mL	300 µL
7	7 mL	350 µL
8	8 mL	400 µL
9	9 mL	450 µL
10	10 mL	500 µL
11	11 mL	550 µL
12	12 mL	600 µL

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

2. Assay Procedure:

(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 other 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 diluted MMAF Tracer Antibody (cat# 30754) to each well. Tap the plate gently.
(6) Seal the plate wells securely, cover with foil or other 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 other 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.

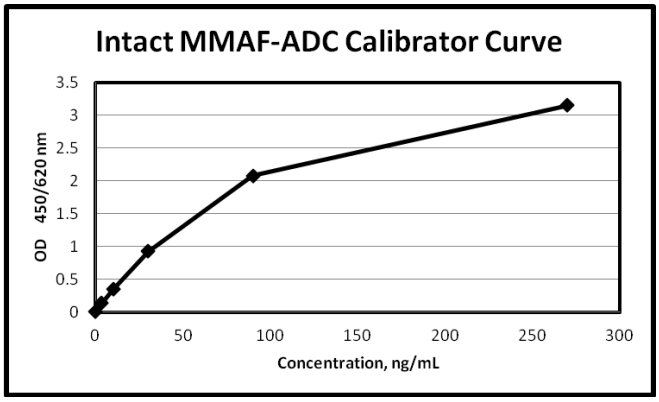
1. Calculate the average absorbance for each pair of duplicate test results.
2. The standard curve is generated by the corrected absorbance of all standard levels on the ordinate against the standard concentration. Appropriate computer assisted data reduction programs should be used for the calculation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

The antibody-MMAF 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 MMAF 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	0.013 0.013	0.013	0.000
3.3 ng/mL	0.138 0.138	0.138	0.125
10.0 ng/mL	0.342 0.346	0.344	0.331
30.0 ng/mL	0.928 0.926	0.927	0.914
90.0 ng/mL	2.067 2.084	2.076	2.063
270.0 ng/mL	3.161 3.139	3.150	3.137



XI. LIMITATION OF THE PROCEDURE

1. This assay requires cell culture, tissue culture samples, serum or plasma sample for testing.
2. For sample values greater than 270 µg/mL, it is recommended to re-assay samples with further dilution with calibrator zero.
3. The kit standards are based on MMAF conjugated antibody or ADC concentration. It is not based on free MMAF concentration. The MMAF-ADC in different linker and DAR may give different curve shift
4. If a higher analytical test sensitivity is desired, a modification by increasing the test sample volume from 25 µl per well to 50 µl or 100 µl per well along with a longer first incubation time period would be very helpful. Please call Epitope Diagnostics for technical support.

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 MMAF-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 0.040 ng/mL. Considering the 1:100 pre-dilution factor of serum or plasma samples, the actual test sensitivity for test sample is about 0.40 pg/ml.

Specificity

This MMAF-ADC ELISA doesn't show any cross reactivity to DM1-ADC. The assay does show cross reactivity to MMAE-ADC.

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

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

Sample #	Mean Value (ng/mL)	CV (%)
1	5.40	1.7
2	18.55	3.6
3	200.95	6.4

The inter-assay precision was validated by measuring two control levels in duplicate in 15 individual assays.

Sample #	Mean Value (ng/mL)	CV (%)
1	19.37	4.1
2	61.61	5.3

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

DILUTION	OBSERVED VALUE (ng/mL)	RECOVERY %
Sample A 1:100	181.7	-
1:2	82.2	90.5
1:4	46.3	101.9
1:8	24.5	108.0
Sample B 1:100	188.6	-
1:2	81.8	86.8
1:4	46.4	98.4
1:8	23.3	98.7

Spike Recovery
Three 1:100 diluted samples are equal volume mixed with standard level 3, 4, 5 and tested. The results are as follows:

Spiked Sample	OBSERVED VALUE (ng/mL)	RECOVERY %
Sample A 1:100	7.1	-
Cal 3: 10 ng/mL	8.1	94.8
Cal 4: 30 ng/mL	17.9	96.4
Cal 5: 90 ng/mL	49.1	101.2
Sample B 1:100	9.3	-
Cal 3: 10 ng/mL	9.3	96.0
Cal 4: 30 ng/mL	18.5	94.0
Cal 5: 90 ng/mL	47.9	96.6

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

Epitope Diagnostics, Inc.
San Diego, CA 92121, USA

Manufacturer	No. of tests
Catalog Number	Keep away from heat and direct sun light
Concentrate	Store at
In Vitro Diagnostic Device	Use by
Read instructions before use	Lot No.

MMAF- ADC 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*

Distribuito in ITALIA da
Li StarFish S.r.l.
Via Cavour, 35
20063 Cernusco S/N (MI)
telefono 02-92150794
fax 02-92157285
info@listarfish.it
www.listarfish.it