

# PRODUCT INFORMATION AND MANUAL

## **human sCD40L Instant ELISA**

**REF** **BMS239INSTCE**

**IVD**

Enzyme-linked immunosorbent assay for quantitative  
detection of human sCD40L.

For in vitro diagnostic use.

Not for therapeutic procedures.

▽ 128 Tests

**CE**

**human sCD40L  
BMS239INSTCE**



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## TABLE OF CONTENTS

1	Intended Use	4
2	Summary	4
3	Principles of the Test	6
4	Reagents Provided	7
5	Storage Instructions	7
6	Specimen Collection	8
7	Materials Required But Not Provided	9
8	Precautions for Use	10
9	Preparation of Reagents and Samples	12
10	Test Protocol	13
11	Calculation of Results	17
12	Limitations	20
13	Performance Characteristics	21
14	Bibliography	26
15	Ordering Information	28
16	Reagent Preparation Summary	29
17	Test Protocol Summary	30
	<b>PRODUKTINFORMATION UND HANDBUCH (Deutsch)</b>	<b>31</b>
1	Mitgelieferte Reagenzien	31
2	Sicherheitsvorkehrungen für den Gebrauch	32
3	Lagerhinweise	33
4	Vorbereitung der Reagenzien	34
5	Testprotokoll	35
	<b>INFORMACIÓN Y MANUAL DEL PRODUCTO (Español)</b>	<b>38</b>
1	Reactivos Suministrados	38
2	Instrucciones de Conservación	39
3	Preparación de los Reactivos	40
4	Protocolo de Ensayo	41
	<b>INFORMATIONS SUR LE PRODUIT ET MANUEL (Français)</b>	<b>44</b>
1	Réactifs Fournis	44
2	Préparation des Réactifs	45

3	Protocolle de Test	46
	<b>INFORMAZIONI SUL PRODOTTO E MANUALE (italiano)</b>	<b>49</b>
1	Reagenti Forniti	49
2	Istruzioni di Conservazione	50
3	Preparazione Dei Reagenti	51
4	Procedura del Test	52

## 1 Intended Use

The human sCD40L Instant ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of soluble human CD40 ligand levels in cell culture supernatants, human serum and plasma. **BMS239 is not suitable for the detection of very low levels of sCD40L in plasma; for this please refer to BMS293, human sCD40L high sensitivity ELISA.**

The human sCD40L Instant ELISA is **for in vitro diagnostic use. Not for use in therapeutic procedures.**

## 2 Summary

CD40 belongs to the TNF receptor superfamily. While the biological role of some of the ligand-receptor pairs in this family still remains obscure, CD40 has proven its importance.(3)

A key role of CD40/CD40ligand interactions in immune activation, particularly in T-cell dependent B cell responses is anticipated. This molecule as well as the other ligands of the family share the property of co-stimulation of T-cell proliferation and are all expressed by activated T-cells.

The programmed cell death has been suggested to be involved in clonal elimination of self-reactive lymphocytes for the normal function of the immune system. Interaction with membrane bound self antigens may eliminate self-reactive nature B cells by apoptosis. Antigen-receptor mediated B cell apoptosis is blocked when a signal is transduced via the CD40 molecule on the B cell surface (10).

Because the ligand of CD40 (CD40L) is expressed on activated T helper cells, B cells may escape from apoptosis and are activated when the immune system interacts with foreign antigens, which are normally able to activate T-helper cells. Thus the CD40 - CD40L interaction plays a central role in the various phases of the B cell response to T-dependent antigens (8).

Taken together, B cells can participate in regulating their own destruction. Protection against Fas-dependent apoptosis afforded by immunoglobulin-receptor engagement may constitute a fail-safe

mechanism that eliminates bystander B cells activated by CD40L - expressing T cells, but ensures survival of antigen-specific B cells (9).

CD40 Ligand is expressed on the surface of activated CD4+ T cells, basophils, and mast cells. Binding of CD40L to its receptor, CD40, on the surface of B cells stimulates B-cell proliferation, adhesion and differentiation. A soluble isoform of CD40L has been shown to exist in the circulation. This soluble molecule is a homotrimer of a 18kDa protein exhibiting full activity in B cell proliferation and differentiation assays, is able to rescue B cells from apoptosis and binds soluble CD40 (4, 6).

CD40L is discussed in relation to a potential role in supporting B cell tumors and it has been discovered that the molecular defect in the X-linked Hyper-IgM-Syndrome (1) is targeted to the CD40 L gene, it is functional involved in B cell hybridomas (5) and chronic lymphocytic leukemia (2) as well as several autoimmune diseases (7).

### 3 Principles of the Test

An anti-human sCD40L monoclonal coating antibody is adsorbed onto microwells. Human sCD40L present in the sample or standard binds to antibodies adsorbed to the microwells; an HRP-conjugated monoclonal anti-human sCD40L antibody binds to human sCD40L captured by the first antibody.

Following incubation unbound enzyme conjugated anti-human sCD40L is removed during a wash step and substrate solution reactive with HRP is added to the wells.

A coloured product is formed in proportion to the amount of human sCD40L present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven human sCD40L standard dilutions and human sCD40L sample concentration determined.

Figure 1

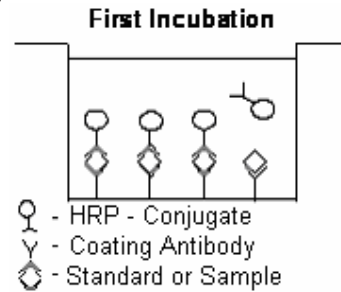


Figure 2

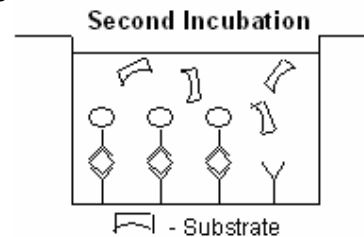
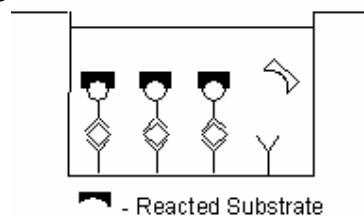


Figure 3



#### 4 Reagents Provided

- 1 aluminium pouch with a **Microwell Plate coated with Monoclonal Antibody** (murine) to human sCD40L, **HRP-Conjugate** (anti-sCD40L monoclonal (murine) antibody) and Sample Diluent, lyophilized
- 2 aluminium pouches with a **human sCD40L Standard curve (coloured)**
- 1 bottle (25 ml) **Wash Buffer Concentrate** 20x (phosphate-buffered saline with 1% Tween 20)
- 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
- 1 vial (12 ml) **Sample Diluent** (Use when an external predilution of the samples is needed)
- 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
- 2 adhesive **Plate Covers**

#### 5 Storage Instructions

Store ELISA plate and Standard curves or whole kit at -20°C. The plate and the standard curves can also be removed, stored at -20°C, remaining kit reagents can be stored between 2° and 8°C. Expiry of the kit and reagents is stated on labels.

The expiry of the kit components can only be guaranteed if the components are stored properly, and if, in case of repeated use of one component, the reagent is not contaminated by the first handling.

## 6 Specimen Collection

Cell culture supernatants, human serum and plasma are suitable for use in the assay.

***BMS239 is not suitable for the detection of very low levels of sCD40L in plasma; for this please refer to BMS293, human sCD40L high sensitivity ELISA.***

Remove the serum or plasma from the clot or red cells as soon as possible after clotting and separation.

Samples containing a visible precipitate must be clarified prior to use in the assay. Do not use grossly hemolyzed or lipemic specimens.

Samples must be stored frozen at -20 °C to avoid loss of bioactive human sCD40L. If samples are to be run within 24 hours, they may be stored at 2° to 8 °C (for sample stability refer to 13).

Avoid repeated freeze-thaw cycles. Prior to assay, frozen serum or plasma should be brought to room temperature slowly and mixed gently.

## 7 Materials Required But Not Provided

- 5 ml and 10 ml graduated pipettes
- 5  $\mu$ l to 1000  $\mu$ l adjustable single channel micropipettes with disposable tips
- 50  $\mu$ l to 300  $\mu$ l adjustable multichannel micropipette with disposable tips
- Multichannel micropipette reservoir
- Beakers, flasks, cylinders necessary for preparation of reagents
- Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
- Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
- Glass-distilled or deionized water
- Statistical calculator with program to perform linear regression analysis

## 8 Precautions for Use

- All chemicals should be considered as potentially hazardous. We therefore recommend that this product is handled only by those persons who have been trained in laboratory techniques and that it is used in accordance with the principles of good laboratory practice. Wear suitable protective clothing such as laboratory overalls, safety glasses and gloves. Care should be taken to avoid contact with skin or eyes. In the case of contact with skin or eyes wash immediately with water. See material safety data sheet(s) and/or safety statements(s) for specific advice.
- Reagents are intended for in vitro diagnostic use and are not for use in therapeutic procedures.
- Do not mix or substitute reagents with those from other lots or other sources.
- Do not use kit reagents beyond expiration date on label.
- Do not expose kit reagents to strong light during storage or incubation.
- Do not pipette by mouth.
- Do not eat or smoke in areas where kit reagents or samples are handled.
- Avoid contact of skin or mucous membranes with kit reagents or specimens.
- Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
- Avoid contact of substrate solution with oxidizing agents and metal.
- Avoid splashing or generation of aerosols.
- In order to avoid microbial contamination or cross-contamination of reagents or specimens which may invalidate the test use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing substrate reagent.

- Glass-distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose specimens and all potentially contaminated materials as they could contain infectious agents. The preferred method of decontamination is autoclaving for a minimum of 1 hour at 121.5°C.
- Liquid wastes not containing acid and neutralized waste may be mixed with sodium hypochlorite in volumes such that the final mixture contains 1.0% sodium hypochlorite. Allow 30 minutes for effective decontamination. Liquid waste containing acid must be neutralized prior to the addition of sodium hypochlorite.

## **9 Preparation of Reagents and Samples**

### **9.1 Wash Buffer**

If crystals have formed in the Wash Buffer Concentrate, warm it gently until they have completely dissolved.

Pour entire contents (25 ml) of the Wash Buffer Concentrate into a clean 500 ml graduated cylinder. Bring to final volume to 500 ml with glass-distilled or deionized water. Mix gently to avoid foaming. The pH of the final solution should adjust to 7.4.

Transfer to a clean wash bottle and store at 2° to 25°C. Please note that Wash Buffer is stable for 30 days.

## 10 Test Protocol

- **Use plate immediately after removal from -20°C!**
  - **Do not wait until pellets have completely dissolved before applying samples - the binding reaction in the standard strips starts immediately after addition of water!**
  - **Do not try to dissolve pellets by pipetting up and down in the wells - some parts of the pellet could stick to the tip creating high variation of results**
  - **Perform the washing step with at least 400 µl of washing buffer as stated in the manual or fill the wells completely - otherwise any pellet residues sticking to the rim of the well will not be removed and create high variation of results**
  - **Allow the washing buffer to sit in the wells for a few seconds before aspiration**
  - **Remove covers of the standard strips carefully in order that all the lyophilised pellets remain in the wells**
- a. Determine the number of Microwell Strips required to test the desired number of samples plus Microwell Strips for blanks and standards (coloured). Each sample, standard, blank, and optional control sample should be assayed in duplicate. Remove extra Microwell Strips from holder and store in foil bag with the desiccant provided at -20°C sealed tightly. Place microwell strips containing the standard curve in position A1/A2 to H1/H2 (see Table 1).
  - b. Add 135 µl of distilled water to the sample wells.
  - c. Add distilled water to all standard and blank wells as indicated on the label of the standard strips (A1, A2 to H1, H2).

Table 1

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Standard 1 (10.00 ng/ml)	Standard 1 (10.00 ng/ml)	Sample 1	Sample 1
<b>B</b>	Standard 2 (5.00 ng/ml)	Standard 2 (5.00 ng/ml)	Sample 2	Sample 2
<b>C</b>	Standard 3 (2.50 ng/ml)	Standard 3 (2.50 ng/ml)	Sample 3	Sample 3
<b>D</b>	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Sample 4	Sample 4
<b>E</b>	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Sample 5	Sample 5
<b>F</b>	Standard 6 (0.32 ng/ml)	Standard 6 (0.32 ng/ml)	Sample 6	Sample 6
<b>G</b>	Standard 7 (0.16 ng/ml)	Standard 7 (0.16 ng/ml)	Sample 7	Sample 7
<b>H</b>	Blank	Blank	Sample 8	Sample 8

- d. Add 15 µl of each **Sample**, in duplicate, to the designated wells and mix the contents.
- e. Cover with a **Plate Cover** and incubate at room temperature (18°C to 25°C) for 2 hours, if available on a microplate shaker at 100 rpm.
- f. Remove **Plate Cover** and empty wells. Wash the microwell strips 3 times with approximately 400 µl Wash Buffer per well with thorough aspiration of microwell contents between washes. Take care not to scratch the surface of the microwells.

After the last wash, tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing or place upside down on a wet absorbent paper for no longer than 15 minutes. Do not allow wells to dry.

- g. Pipette 100 µl of **TMB Substrate Solution** to all wells, including the blank wells.
- h. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes. Avoid direct exposure to intense light.

**The colour development on the plate should be monitored and the substrate reaction stopped (see point i. of this protocol) before positive wells are no longer properly recordable.**

It is recommended to add the stop solution when the highest standard has developed a dark blue colour.

Alternatively the colour development can be monitored by the ELISA reader at 620 nm. The substrate reaction should be stopped as soon as the standard 1 has reached an OD of 0.6 – 0.65.

- i. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well, including the blank wells. It is important that the Stop Solution is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Stop Solution is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- j. Read absorbance of each microwell on a spectro-photometer using 450 nm as the primary wave length (optionally 620 nm as the reference wave length; 610 nm to 650 nm is acceptable). Blank the plate reader according to the manufacturer's instructions by using the

blank wells. Determine the absorbance of both the samples and the human sCD40L standards.

## 11 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human sCD40L concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating human sCD40L for each sample, first find the mean absorbance value on the ordinate and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the abscissa and read the corresponding human sCD40L concentration.
- **\*Samples have been diluted 1:5, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 5).**
- It is suggested that each testing facility establishes a control sample of known human sCD40L concentration and runs this additional control with each assay. If the values obtained are not within the expected range of the control, the assay results may be invalid.
- A representative standard curve is shown in Figure 4. This curve cannot be used to derive test results. Every laboratory must prepare a standard curve for each group of microwell strips assayed.

\* N.B: There is a common dilution factor for samples due to the conjugate which must then be included in the calculation. The samples contribute 75 µl to the final volume per well. These 75 µl are composed of 60 µl of sample diluent plus 15 µl of the sample. This is a 1:5 dilution. The remaining 75 µl to give 150 µl are due to the addition of 75 µl conjugate to all wells.

60 µl sample diluent and 75 µl conjugate results in 135 µl reconstitution volume, addition of 15 µl sample (60 µl + 15 µl = 1:5 dilution)

Figure 4

Representative standard curve for human sCD40L Instant ELISA. Human sCD40L was diluted in serial 2-fold steps in Sample Diluent, each symbol represents the mean of 3 parallel titrations. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

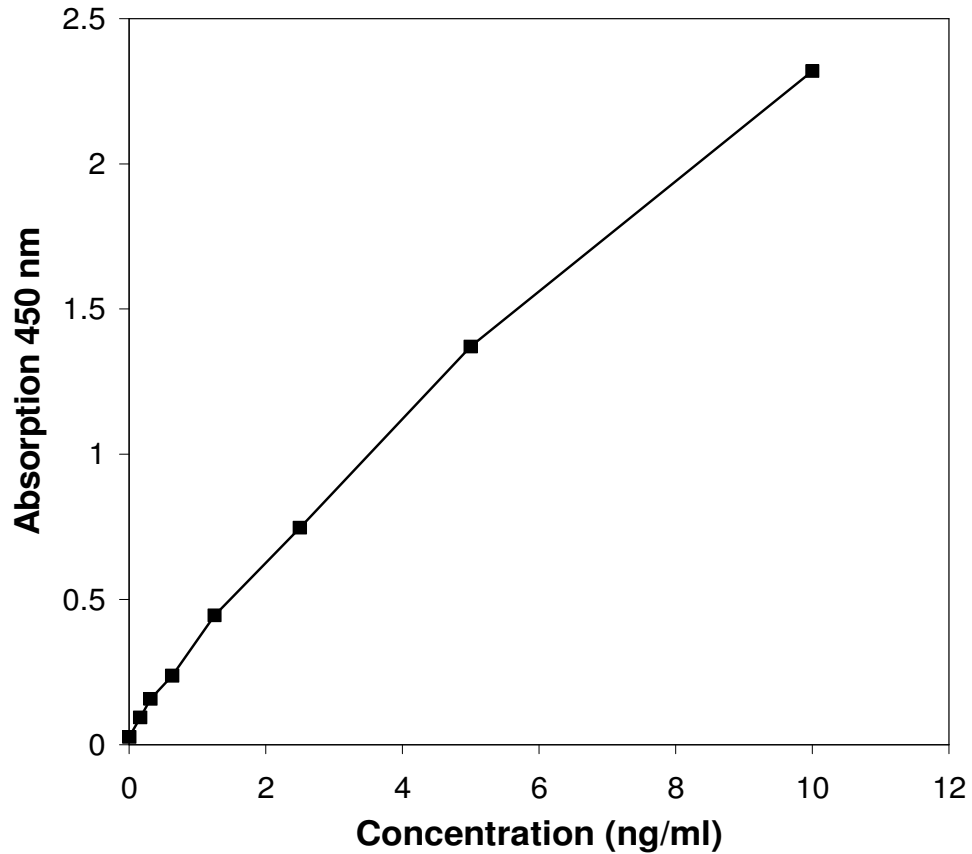


Table 2

Typical data using the human sCD40L INSTANT ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

Standard	human sCD40L Concentration (ng/ml)	O.D. (450 nm)	O.D. Mean	C.V. (%)
1	10.00	2.301	2.319	1.1
	10.00	2.336		
2	5.00	1.396	1.371	2.6
	5.00	1.346		
3	2.50	0.728	0.747	3.6
	2.50	0.766		
4	1.25	0.461	0.445	5.2
	1.25	0.428		
5	0.63	0.245	0.237	5.1
	0.63	0.228		
6	0.31	0.155	0.157	1.4
	0.31	0.158		
7	0.16	0.089	0.093	6.1
	0.16	0.097		
Blank	0	0.023	0.026	
	0	0.029		

## 12 Limitations

- Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
- Bacterial or fungal contamination of either screen samples or reagents or cross-contamination between reagents may cause erroneous results.
- Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
- Improper or insufficient washing at any stage of the procedure will result in either false positive or false negative results. Empty wells completely before dispensing fresh wash solution, fill with Wash Buffer as indicated for each wash cycle and do not allow wells to sit uncovered or dry for extended periods.
- The use of radioimmunotherapy has significantly increased the number of patients with human anti-mouse IgG antibodies (HAMA). HAMA may interfere with assays utilizing murine monoclonal antibodies leading to both false positive and false negative results. Serum samples containing antibodies to murine immunoglobulins can still be analysed in such assays when murine immunoglobulins (serum, ascitic fluid, or monoclonal antibodies of irrelevant specificity) are added to the Sample.

## **13 Performance Characteristics**

### **13.1 Sensitivity**

The limit of detection of human sCD40L defined as the analyte concentration resulting in an absorbance significantly higher than that of the dilution medium (mean plus 2 standard deviations) was determined to be 0.062 ng/ml (mean of 6 independent assays).

### **13.2 Reproducibility**

#### **13.2.1 Intra-assay**

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 5 serum samples containing different concentrations of human sCD40L. 2 standard curves were run on each plate. Data below show the mean human sCD40L concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 6.8%.

Table 3

The Mean human sCD40L concentration and the coefficient of variation for each sample.

Positive Sample	Experiment	human sCD40L Concentration (ng/ml)	Coefficient of Variation (%)
1	1	8.8	0.8
	2	7.3	2.7
	3	7.4	5.6
2	1	14.7	13.3
	2	11.2	2.7
	3	10.6	5.6
3	1	16.5	13.4
	2	12.9	9.6
	3	13.3	11.1
4	1	19.3	1.4
	2	15.3	3.6
	3	14.6	16.2
5	1	38.7	3.4
	2	30.0	5.6
	3	32.7	7.6

### 13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments by 3 technicians. Each assay was carried out with 6 replicates of 5 serum samples containing different concentrations of human sCD40L. 2 standard curves were run on each plate. Data below (see Table 4) show the mean human sCD40L concentration and the coefficient of variation calculated on 18 determinations of each sample. The calculated overall coefficient of variation was 14.2%.

Table 4

The mean human sCD40L concentration and the coefficient of variation calculated on 18 determinations of each sample.

Sample	human sCD40L Concentration (ng/ml)	Coefficient of Variation (%)
1	7.9	10.8
2	12.2	18.0
3	14.3	13.9
4	16.4	15.3
5	33.8	13.1

### 13.3 Spike Recovery

The spike recovery was evaluated by spiking 4 levels of human sCD40L into normal human sera. Recoveries were determined in 3 independent experiments with 6 replicates each. The unspiked serum was used as blank in these experiments. Average recovery ranged from 76 to 87% with an overall mean recovery of 82%.

### 13.4 Dilution Parallelism

4 serum samples with different levels of human sCD40L were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged between 85% and 102% with an overall mean recovery of 92%.

### 13.5 Sample Stability

#### 13.5.1 Freeze-Thaw Stability

Aliquots of serum samples (unspiked or spiked) were stored at -20 °C and thawed 5 times, and the human sCD40L levels determined. There was no significant loss of human sCD40L immunoreactivity by freezing and thawing.

#### 13.5.2 Storage Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20 °C, 2-8 °C, room temperature (RT) and at 37 °C, and the human sCD40L level determined after 24 h. As shown in Table 5, there was a significant loss of human sCD40L immunoreactivity during storage at RT and 37 °C.

Table 5

Storage Temperature	sCD40L Serum		sCD40L Spike	
	Concentration (ng/ml)	Recovery (%)	Concentration (ng/ml)	Recovery (%)
- 20 °C	5.7	100	5.8	100
2 - 8 °C	4.7	82	5.6	96
RT	0.7	13	3.3	57
37 °C	0.0	0	0.2	4

### 13.6 Comparison of Serum and Plasma

From eight individuals, serum as well as EDTA plasma, citrate plasma, and heparin plasma obtained at the same time point were evaluated. It clearly turned out that plasma preparations give results that do not correlate with the respective serum data.

### 13.7 Specificity

To define the specificity of this ELISA several proteins were tested for cross reactivity. There was no cross reactivity observed.

### **13.8 Expected Values**

A panel of 40 randomly selected sera from healthy blood donors (males and females) was tested for human sCD40L. The detected human sCD40L levels ranged between 0.03 and 3.98 ng/ml with a mean level of 2.13 ng/ml and a standard deviation of  $\pm 1.0$  ng/ml.

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For literature update refer to **[www.bendermedsystems.com/44.html](http://www.bendermedsystems.com/44.html)**

## 15 Ordering Information

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**Cat.No. BMS239INSTCE human sCD40L INSTANT ELISA**

## **16 Reagent Preparation Summary**

### **16.1 Wash Buffer**

Add **Wash Buffer Concentrate** 20 x (25 ml) to 475 ml distilled water

## 17 Test Protocol Summary

- Place standard strips in position A1/A2 to H1/H2.
- Add 135 µl **distilled water** to sample wells.
- Add **distilled water**, in duplicate, to all standard and blank wells as indicated on the label of the standard strips.
- Add 15 µl **Sample** to designated wells.
- Cover microwell strips and incubate 2 hours at room temperature (18° to 25°C) if available on a microplate shaker at 100 rpm.
- Empty and wash microwell strips 3 times with 400 µl **Wash Buffer**.
- Add 100 µl of **TMB Substrate Solution** to all wells including blank wells.
- Incubate the microwell strips for about 10 minutes at room temperature (18° to 25°C).
- Add 100 µl **Stop Solution** to all wells including blank wells.
- Blank microwell reader and measure colour intensity at 450 nm.

**Note: Samples have been diluted 1:5, thus the concentration read from the standard curve must be multiplied by the dilution factor (x 5).**

# PRODUKTINFORMATION UND HANDBUCH (Deutsch)

## 1 Mitgelieferte Reagenzien

- 1 Aluminiumbeutel mit Mikrotiterplatte, beschichtet mit monoklonalem Antikörper (von der Maus) gegen humanes sCD40L, HRP-Konjugat (monoklonaler Anti-sCD40L Antikörper von der Maus) und Verdünnungslösung, lyophilisiert.
- 2 Aluminiumbeutel mit einer human sCD40L-Standardkurve (farbig)
- 1 Flasche (25 ml) Waschpufferkonzentrat 20x (PBS mit 1% Tween 20)
- 1 Fläschchen (15 ml) Substratlösung (Tetramethylbenzidin)
- 1 Fläschchen (12 ml) Verdünnungslösung (Verwendung zur Vorverdünnung von Proben)
- 1 Fläschchen (15 ml) Stopplösung (1 M Phosphorsäure)
- 2 Klebefolien

## 2 Sicherheitsvorkehrungen für den Gebrauch

- Alle enthaltenen Reagenzien sollten als potenziell gefährlich betrachtet werden. Daher wird empfohlen, dass dieses Produkt nur von Personen mit labortechnischer Erfahrung und in Übereinstimmung mit GLP Richtlinien verwendet wird. Passende Schutzbekleidung, wie Labormäntel, Sicherheitsbrillen und Laborhandschuhe müssen getragen werden. Vermeiden Sie jeden Kontakt der Reagenzien mit Haut oder Augen. Im Falle des Kontaktes von Reagenzien mit Haut oder Augen, sofort mit Wasser spülen. Bitte entnehmen Sie weitere spezifische Hinweise den Sicherheitsdatenblättern und/oder den Sicherheitsbestimmungen.
- Die Reagenzien sind ausschließlich für Diagnosezwecke bestimmt und nicht für den Einsatz bei Therapien.
- Reagenzien aus verschiedenen Chargen oder anderer Herkunft nicht mischen oder untereinander austauschen.
- Verwenden Sie die Kitreagenzien nicht nach dem Ablaufdatum (siehe Etikett).
- Setzen Sie die Kitreagenzien während der Lagerung oder Inkubation keiner starken Lichteinstrahlung aus.
- Nicht mit dem Mund pipettieren.
- In Bereichen, in denen mit Kitreagenzien oder Proben hantiert wird, nicht essen, trinken oder rauchen.
- Vermeiden Sie den Kontakt der Haut/Schleimhäute mit Kitreagenzien/Proben.
- Tragen Sie während des Hantierens mit Kitreagenzien oder Proben geeignete Gummi- oder Einweghandschuhe.
- Vermeiden Sie den Kontakt zwischen Substratlösung und Oxidationsmitteln/Metallen.
- Vermeiden Sie Spritzer oder Bildung von Aerosolen.
- Zur Vermeidung von Kontamination mit Mikroben oder Kreuzkontamination der Reagenzien oder Proben, die den Test

ungültig machen könnten, verwenden Sie Einwegpipettenspitzen und/oder Einwegpipetten.

- Verwenden Sie saubere, geeignete Reagenzgefäße für das Dispensieren von Konjugat und Substratreagenzien.
- Säureeinwirkung inaktiviert das Konjugat.
- Für die Reagenzherstellung muss destilliertes oder entionisiertes Wasser verwendet werden.
- Die Substratlösung muss vor der Verwendung auf Raumtemperatur gebracht werden.
- Dekontaminieren und entsorgen Sie Proben sowie alle möglicherweise kontaminierten Materialien so, als ob sie Infektionserreger enthalten könnten. Die bevorzugte Dekontaminationsmethode ist Autoklavieren für mind. eine Stunde bei 121,5°C.
- Flüssige Abfälle, die kein Säure enthalten, sowie neutralisierte Abfälle werden zur Dekontamination mit Natrium Hypochlorit versetzt (Endkonzentration von Natrium Hypochlorit 1.0%). Nach 30 min ist eine effektive Dekontamination erreicht. Flüssige Abfälle, die Säure enthalten, müssen vor der Dekontamination neutralisiert werden.

### **3 Lagerhinweise**

Lagern Sie die ELISA Platte und Standardkurven oder den ganzen Kit bei –20°C. Die Platte und Standardkurven können auch herausgenommen und bei –20°C gelagert werden, die verbleibenden Reagenzien können bei 2-8°C gelagert werden. Das Ablaufdatum des Kits und der Reagenzien ist auf den Etiketten angegeben.

Haltbarkeit des Kits und der Komponenten kann nur bei sachgemäßer Lagerung garantiert werden, sowie bei mehrfacher Verwendung nur dann, wenn die Reagenzien bei der ersten Verwendung nicht kontaminiert wurden.

## **4 Vorbereitung der Reagenzien**

### **4.1 Waschpuffer**

Sollten sich im Waschpufferkonzentrat Kristalle gebildet haben, erwärmen Sie es vorsichtig bis zur vollständigen Auflösung der Kristalle.

Leeren Sie den gesamten Inhalt (25 ml) des Waschpufferkonzentrats in einen sauberen 500-ml-Messzylinder. Füllen Sie mit destilliertem oder entionisiertem Wasser auf, bis ein Endvolumen von 500 ml erreicht ist. Vorsichtig mischen, um Schäumen zu vermeiden. Der pH-Wert der Endlösung sollte bei 7,4 liegen.

In eine saubere Waschflasche umfüllen und bei 2° bis 25 °C lagern. Bitte beachten Sie, dass der Waschpuffer 30 Tage haltbar ist.

## 5 Testprotokoll

- Verwenden Sie die Platte sofort nach Entnahme von  $-20^{\circ}\text{C}$ !
  - Warten Sie mit dem Auftragen der Proben nicht bis zur vollständigen Auflösung der Lyophilisate – die Bindereaktion in den Standardstreifen beginnt sofort nach Beigabe von Wasser!
  - Versuchen Sie nicht, die Lyophilisate mittels Auf- und Abpipettieren in den Vertiefungen aufzulösen – Teile des Lyophilisats könnten dabei an der Spitze hängen bleiben und starke Abweichungen der Ergebnisse bewirken.
  - Führen Sie den Waschschrift mit mind.  $400\ \mu\text{l}$  Waschpuffer durch wie in der Anleitung beschrieben, oder füllen Sie die Vertiefungen vollständig – andernfalls werden allfällige Lyophilisatrückstände am Rand der Vertiefung nicht entfernt und bewirken starke Abweichungen der Ergebnisse.
  - Entfernen Sie die Abdeckungen der Standardstreifen vorsichtig, sodass alle Lyophilisate in den Vertiefungen bleiben.
- a. Bestimmen Sie die Anzahl der **Mikrowellstreifen** die für das Testen der gewünschten Anzahl von Proben benötigt werden sowie die **Mikrowellstreifen** für Blindproben und Standards (**farbig**). Probe, Standard, Blindprobe und optionale Kontrollprobe immer jeweils doppelt testen. Entfernen Sie die zusätzlichen **Mikrowellstreifen** von der Halterung und bewahren Sie diese mit dem mitgelieferten Trockenmittel in dem Folienbeutel fest verschlossen bei  $-20^{\circ}\text{C}$  auf. Bringen Sie die Mikrowellstreifen mit der Standardkurve in Position A1/A2 bis H1/H2 (siehe Table 6).
- b. Pipettieren Sie in alle Probenvertiefungen  $135\ \mu\text{l}$  destilliertes Wasser.
- c. Pipettieren Sie destilliertes Wasser in alle Standardvertiefungen und Blindprobenvertiefungen wie auf dem Etikett der Standardstreifen angegeben (A1, A2 bis H1, H2).

Table 6

Diagramm mit Beispiel für die Anordnung von Blindproben, Standards und Proben in den Mikrowellstreifen:

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Standard 1 (10.00 ng/ml)	Standard 1 (10.00 ng/ml)	Probe 1	Probe 1
<b>B</b>	Standard 2 (5.00 ng/ml)	Standard 2 (5.00 ng/ml)	Probe 2	Probe 2
<b>C</b>	Standard 3 (2.50 ng/ml)	Standard 3 (2.50 ng/ml)	Probe 3	Probe 3
<b>D</b>	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Probe 4	Probe 4
<b>E</b>	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Probe 5	Probe 5
<b>F</b>	Standard 6 (0.32 ng/ml)	Standard 6 (0.32 ng/ml)	Probe 6	Probe 6
<b>G</b>	Standard 7 (0.16 ng/ml)	Standard 7 (0.16 ng/ml)	Probe 7	Probe 7
<b>H</b>	Blindprobe	Blindprobe	Probe 8	Probe 8

- d. Pipettieren Sie je **15 µl von jeder Probe** (Doppelbestimmung) in die Probenvertiefungen und mischen Sie den Inhalt durch.
- e. Mit einer **Klebefolie** abdecken und bei Raumtemperatur (18° bis 25°C) für 2 Stunden inkubieren, wenn möglich auf einem Schüttler bei 100 U/min.
- f. Entfernen Sie die **Klebefolie** und entleeren Sie die Vertiefungen  
Waschen Sie die Mikrowellstreifen 3 mal mit ca. **400 µl Waschpuffer** pro Vertiefung; zwischen den Waschgängen den Inhalt der Vertiefungen gründlich absaugen. Achten Sie darauf, die Oberfläche der Vertiefungen nicht zu zerkratzen.

- g. Leeren Sie die Vertiefungen nach dem letzten Waschschrift und klopfen Sie die Mikrowellstreifen auf einem Saug- oder Papiertuch aus, um überschüssigen Waschpuffer zu entfernen. Verwenden Sie die Mikrowellstreifen sofort nach dem Waschen, oder legen Sie diese für maximal 15 Minuten umgedreht auf ein nasses Saugtuch. Lassen Sie die Vertiefungen nicht austrocknen
- h. Pipettieren Sie in alle Vertiefungen, einschließlich der Blindprobenvertiefungen, 100 µl **TMB-Substratlösung**.
- i. Inkubieren Sie die Mikrowellstreifen bei Raumtemperatur (18° bis 25 °C) für ca. 10 Minuten. Vermeiden Sie direkte, starke Lichteinstrahlung. Die Farbentwicklung innerhalb der einzelnen Vertiefungen muss beobachtet und die Substratreaktion gestoppt werden, bevor die gefärbten wells nicht mehr richtig gemessen können. Es wird empfohlen, die Stopplösung zuzugeben, wenn der höchste Standardpunkt eine dunkelblaue Farbe angenommen hat. Alternativ kann die Farbentwicklung auch mit einem Photometer bei 620 nm verfolgt werden. Die Substratreaktion sollte gestoppt werden, wenn der höchste Standardpunkt eine OD von 0,6-0,65 erreicht.
- j. Stoppen Sie die Enzymreaktion durch rasches Pipettieren von 100 µl Stopplösung in jede Vertiefung, einschließlich der Blindprobenvertiefungen. Für eine vollständige Inaktivierung der Enzyme ist es wichtig, die Stopplösung rasch und gleichmäßig in die Vertiefungen zu verteilen. Die OD Werte müssen sofort nach Beigabe der Stopplösung oder innerhalb einer Stunde nach Lagerung der Mikrowellstreifen in Dunkelheit bei 2-8 °C gemessen werden.
- k. Messen Sie die Absorption jeder Vertiefung mit einem Spektrophotometer, verwenden Sie dabei 450 nm als primäre Wellenlänge (optional 620 nm als Referenzwellenlänge; 610 nm bis 650 nm sind möglich). Stellen Sie das Plattenmessgerät nach Anleitung des Herstellers und unter Verwendung der Blindprobenvertiefungen auf den Leerwert ein Bestimmen Sie die Absorption der Proben wie auch der human sCD40L-Standards.

# INFORMACIÓN Y MANUAL DEL PRODUCTO (Español)

## 1 Reactivos Suministrados

- 1 bolsa de aluminio con una placa de pocillos recubiertos con anticuerpos monoclonales (murinos) anti-sCD40L humano, diluyente para muestras y conjugado HRP [anticuerpos (murinos) monoclonales anti-sCD40L], liofilizado
- 2 bolsas de aluminio con una curva de valoración human sCD40L (en color)
- 1 frasco (25 ml) de concentrado de tampón de lavado 20x (PBS con Tween 20 al 1%)\*
- 1 vial (15 ml) de solución de sustrato (tetrametil-bencidina)
- 1 vial (12 ml) de diluyente de muestras
- 1 vial (15 ml) de solución de parada (ácido fosfórico 1M)
- 2 tapas para placas, adhesives

## 2 Instrucciones de Conservación

Conservar la placa de ELISA o el kit completo a  $-20^{\circ}\text{C}$ . También se puede sacar la placa para conservarla a  $-20^{\circ}\text{C}$  y conservar los demás reactivos del kit a una temperatura comprendida entre  $2$  y  $8^{\circ}\text{C}$ . En las etiquetas figuran las fechas de caducidad del kit y de los reactivos. Sólo se podrá garantizar la fecha de caducidad de los componentes del *kit* si se conservan adecuadamente y, en caso de uso reiterado de un mismo componente, si el reactivo no queda contaminado en la primera manipulación.

### **3 Preparación de los Reactivos**

#### **3.1 Tampón de lavado**

Si en el concentrado de tampón de lavado se han formado cristales, caliente suavemente hasta su completa disolución.

Vierta todo el contenido (25 ml) del Concentrado de tampón de lavado en un matraz aforado de 500 ml limpio. Enrase en matraz con agua destilada o desionizada. Mezcle suavemente para evitar la formación de espuma. Ajuste el pH de la solución final a 7,4.

Transfiera la solución a un frasco de lavado limpio y consérvela a una temperatura entre 2 °C y 25 °C. El Tampón de lavado permanece estable durante 30 días.

#### 4 Protocolo de Ensayo

- **Utilice la placa inmediatamente después de extraerla del congelador a -20 °C**
  - **Al aplicar las muestras, no espere a la disolución total de las microcápsulas ya que la reacción de unión a las tiras de patrón se inicia inmediatamente después de añadir el agua.**
  - **No intente disolver las microcápsulas pipeteando por los pocillos ya que algunas partes de las microcápsulas podrían quedar adheridas a la punta y alterar considerablemente los resultados**
  - **En la etapa de lavado, emplee al menos 400 µl de tampón de lavado como se indica en el manual o llene los pocillos totalmente ya que, de lo contrario, podrían quedar restos de microcápsulas en los bordes de los pocillos que alterarían considerablemente los resultados**
  - **Retire las tapas de las tiras de patrón con cuidado para que todas las microcápsulas liofilizadas permanezcan en los pocillos**
- a. Determine el número de tiras necesarias para analizar el número deseado de muestras y además añada las **tiras** para blancos y patrones (**de color**). Todas las muestras, patrones, blancos y las posibles muestras de control deben ser analizadas por duplicado. Retire del soporte las **tiras** sobrantes y consérvelas, junto con el desecante suministrado en una bolsa metalizada y cerrada herméticamente, a una temperatura de -20° C. Coloque las tiras que contienen la curva de valoración en las posiciones A1/A2 a H1/H2 (véase la Table 7).
  - b. Añada 135 µl de agua destilada a los pocillos con muestras.
  - c. Añada agua destilada a los pocillos del patrón y del blanco como se indica en la etiqueta de las tiras de patrón (A1, A2 a H1, H2).
  - d. Por duplicado, añada 15 µl de cada **muestra** a los pocillos designados y mezcle los contenidos.

Table 7

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Patrón 1 (10.00 ng/ml)	Patrón 1 (10.00 ng/ml)	Muestra 1	Muestra 1
<b>B</b>	Patrón 2 (5.00 ng/ml)	Patrón 2 (5.00 ng/ml)	Muestra 2	Muestra 2
<b>C</b>	Patrón 3 (2.50 ng/ml)	Patrón 3 (2.50 ng/ml)	Muestra 3	Muestra 3
<b>D</b>	Patrón 4 (1.25 ng/ml)	Patrón 4 (1.25 ng/ml)	Muestra 4	Muestra 4
<b>E</b>	Patrón 5 (0.63 ng/ml)	Patrón 5 (0.63 ng/ml)	Muestra 5	Muestra 5
<b>F</b>	Patrón 6 (0.31 ng/ml)	Patrón 6 (0.31 ng/ml)	Muestra 6	Muestra 6
<b>G</b>	Patrón 7 (0.16 ng/ml)	Patrón 7 (0.16 ng/ml)	Muestra 7	Muestra 7
<b>H</b>	blanco	blanco	Muestra 8	Muestra 8

- e. Cubra la **placa con una tapa** e incúbela a temperatura ambiente (18°C - 25°C) durante 2 horas (en un agitador mecánico a 100 rpm, si es posible).
- f. Retire la **tapa** y vacíe los pocillos. Lave 3 veces las tiras con aproximadamente 400 µl de tampón de lavado por cada pocillo, aspirando completamente el contenido de los pocillos entre cada lavado. Evite rayar la superficie de los pocillos.
- g. Tras el último lavado, golpee suavemente las tiras contra un papel absorbente o una toallita de papel para eliminar el exceso de tampón de lavado. Utilice las tiras inmediatamente después de lavadas o bien colóquelas boca abajo sobre un papel absorbente húmedo durante como máximo 15 minutos. No deje secar los pocillos.

- h. Pipetee 100  $\mu$ l de **solución de sustrato TMB** y viértalos en todos los pocillos, incluidos los del blanco.
- i. Incube las tiras a temperatura ambiente (18 °C - 25 °C) durante aproximadamente 10 minutos. Evite la exposición directa a la luz intensa. **Deben monitorizarse los valores DO de la placa para detener la reacción del sustrato antes de que deje de ser posible registrar correctamente los pocillos positivos.** Se recomienda añadir la solución de parada cuando el estándar más alto presente un color azul oscuro. Alternativamente el desarrollo de color puede ser monitorizado con un lector de placas de ELISA a 620 nm. La reacción del sustrato debería ser parada cuando este estándar alcance una OD entre 0.6 y 0.65.
- j. Detenga la reacción enzimática pipeteando rápidamente 100  $\mu$ l de **solución de parada** en cada pocillo, incluidos los del blanco. Es importante dispensar la solución de parada de forma rápida y uniforme en todos los pocillos para inactivar totalmente la enzima. Los resultados deben leerse inmediatamente después de añadir la solución de parada o, como máximo, en el plazo de 1 hora si las tiras se conservan a una temperatura entre 2 - 8 °C en un lugar oscuro.
- k. Lea la absorbancia de cada pocillo en un espectrofotómetro utilizando 450 nm como longitud de onda principal (opcionalmente 620 nm como longitud de onda de referencia; los valores comprendidos entre 610 nm y 650 nm son aceptables). Utilizando los pocillos de blanco, haga el blanco del lector de placas de acuerdo con las instrucciones del fabricante. Determine la absorbancia de las muestras y de los patrones human sCD40L.

# INFORMATIONS SUR LE PRODUIT ET MANUEL (Français)

## 1 Réactifs Fournis

- 1 pochette en aluminium contenant une plaque de microtitration recouverte d'anticorps monoclonaux (murins) anti-sCD40L humaine, un diluant d'échantillon et le conjugué HRP (anticorps monoclonaux murins anti-sCD40L), lyophilisés
- 2 pochettes en aluminium contenant une courbe étalon human sCD40L (colorée)
- 1 flacon (25 ml) de tampon de lavage concentré 20 x (tampon phosphate avec du Tween 20 1%)
- 1 flacon (15 ml) de solution de substrat (tétraméthyle-benzidine)
- 1 flacon (12 ml) de diluant d'échantillon
- 2 flacon (15 ml) de solution d'arrêt (acide phosphorique 1 M)
- 2 Couvre-plaques adhésifs

## **2 Préparation des Réactifs**

### **2.1 Tampon de lavage**

Si des cristaux se sont formés dans le concentré de Tampon de lavage, chauffer doucement ce dernier jusqu'à leur dissolution totale.

Verser tout le contenu (25 ml) du Concentré de Tampon de lavage dans un cylindre gradué propre de 500 ml. Porter le volume final à 500 ml avec de l'eau distillée dans un alambic en verre ou désionisée.

Mélanger doucement pour éviter la formation de mousse. Le pH de la solution finale doit être de 7.4.

Transférer le tout dans une bouteille de lavage et conserver à une température comprise entre 2° et 25°C. Noter que le Tampon de lavage reste stable pendant 30 jours.

### 3 Protocole de Test

- **Utiliser la plaque immédiatement après son retrait d'un environnement à -20 °C !**
  - **Ne pas attendre que les pastilles soient complètement dissoutes pour appliquer les échantillons. La réaction de liaison dans les barrettes étalons commence immédiatement après l'ajout d'eau !**
  - **Ne pas essayer de dissoudre les pastilles en pipetant de haut en bas dans les puits. Certains fragments des pastilles pourraient se coller à l'embout et induire une forte variation des résultats.**
  - **Procéder à l'étape de lavage avec au moins 400 µl de tampon de lavage comme indiqué dans le manuel ou remplir complètement les puits. Dans le cas contraire, tous les résidus de pastilles collés au bord du puits ne seraient pas éliminés et entraîneraient une forte variation des résultats**
  - **Retirer délicatement les couvre-plaques des barrettes étalons de manière à ce que toutes les pastilles lyophilisées restent dans les puits**
- a. Déterminer le nombre de barrettes de puits de microtitration nécessaires pour tester le nombre voulu d'échantillons plus les **barrettes** nécessaires aux blancs et aux étalons (**colorés**). Chaque échantillon, étalon, blanc et contrôle (facultatif) doit être testé en double. Retirer les **barrettes de microtitration** inutiles du support et les stocker à -20°C dans une pochette hermétiquement refermée, avec le dessiccatif fourni. Placer les barrettes de puits de microtitration contenant la courbe étalon en position A1/A2 à H1/H2 (voir la Table 8).
- b. Ajouter 135 µl d'eau distillée dans les puits d'échantillon.
- c. Ajouter de l'eau distillée comme indiqué sur l'étiquette à tous les puits de standard et "blank" (A1, A2 à H1, H2)

Table 8

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Étalon 1 (10.00 ng/ml)	Étalon 1 (10.00 ng/ml)	Échantillon 1	Échantillon 1
<b>B</b>	Étalon 2 (5.00 ng/ml)	Étalon 2 (5.00 ng/ml)	Échantillon 2	Échantillon 2
<b>C</b>	Étalon 3 (2.50 ng/ml)	Étalon 3 (2.50 ng/ml)	Échantillon 3	Échantillon 3
<b>D</b>	Étalon 4 (1.25 ng/ml)	Étalon 4 (1.25 ng/ml)	Échantillon 4	Échantillon 4
<b>E</b>	Étalon 5 (0.63 ng/ml)	Étalon 5 (0.63 ng/ml)	Échantillon 5	Échantillon 5
<b>F</b>	Étalon 6 (0.31 ng/ml)	Étalon 6 (0.31 ng/ml)	Échantillon 6	Échantillon 6
<b>G</b>	Étalon 7 (0.16 ng/ml)	Étalon 7 (0.16 ng/ml)	Échantillon 7	Échantillon 7
<b>H</b>	Blind Échantillon	Blind Échantillon	Échantillon 8	Échantillon 8

- d. Ajouter 15 µl de chaque **échantillon**, en double, dans les puits assignés et mélanger le contenu.
- e. Recouvrir avec un **couvre-plaque** et incuber à température ambiante (entre 18 et 25 °C) pendant 2 heures, si possible sur un agitateur rotateur réglé à 100 tr/min.
- f. Retirer le **couvre-plaque** et vider les puits. Laver 3 fois les barrettes de puits de microtitration avec environ 400 µl de tampon de lavage pour chaque puits, en aspirant complètement le contenu des puits entre les lavages. Veiller à ne pas rayer la surface des puits de microtitration.
- g. Après le dernier lavage, vider les barrettes de puits et les tapoter sur un tampon absorbant ou une serviette en papier pour éliminer l'excès

de tampon de lavage. Utiliser les barrettes de micropuits immédiatement après le lavage ou les placer renversées sur un papier absorbant pendant 15 minutes au maximum. Ne pas laisser sécher les puits.

- h. Pipeter 100  $\mu$ l de **solution de substrat TMB** dans chaque puits, y compris les puits de blanc.
- g. Incuber les puits de microtitration à température ambiante (entre 18 et 25 C) pendant environ 10 minutes. Éviter toute exposition directe à une source de lumière intense.

**Les valeurs de densité optique au niveau de la plaque doivent être surveillées et la réaction du substrat stoppée avant que les puits positifs ne soient plus correctement mesurables.**

- i. Il est recommandé d'ajouter la solution stop quand une couleur bleu sombre s'est développée dans le point le plus haut de la gamme standard. Une autre alternative consiste à suivre le développement de la couleur en lecteur ELISA à 620 nm. La réaction du substrat doit être arrêtée dès que la DO atteint 0.6 à 0.65
- j. Arrêter la réaction enzymatique en pipetant rapidement 100  $\mu$ l de **solution d'arrêt** dans chaque puits, y compris les puits de blanc. Il est important que la solution d'arrêt soit répandue rapidement et uniformément dans les puits pour inactiver complètement l'enzyme. Les résultats doivent être lus immédiatement après l'ajout de la solution d'arrêt ou dans l'heure qui suit si les barrettes de microtitration sont conservées à l'obscurité entre 2 et 8 °C.
- k. Lire l'absorbance de chaque puits sur un spectrophotomètre avec 450 nm comme longueur d'onde primaire (éventuellement 620 nm comme longueur d'onde de référence; 610 à 650 nm sont acceptables). Mesurer le blanc du lecteur de plaque conformément aux instructions du fabricant, en utilisant les puits de blanc. Déterminer l'absorbance des échantillons et des étalons human sCD40L.

# INFORMAZIONI SUL PRODOTTO E MANUALE (italiano)

## 1 Reagenti Forniti

- 1 bustina di alluminio contenente una piastra micropozzetti rivestita con anticorpo monoclonale (murino) anti sCD40L umano, diluente campione e HRP-coniugato (anticorpo monoclonale (murino) anti-sCD40L), liofilizzato
- 2 bustine di alluminio ognuna con una curva degli étalon human sCD40L (colorata)
- 1 bottiglia (25 ml) con tampone di lavaggio concentrato 20x (soluzione salina tamponata con 1% Tween 20)\*
- 1 flaconcino (15 ml) di soluzione del substrato (tetrametilbenzidina)
- 1 flaconcino (12 ml) con diluente campione
- 1 flaconcino (15 ml) di soluzione bloccante (acido fosforico 1M)
- 2 copripiastra adesivi

## **2 Istruzioni di Conservazione**

Conservare la piastra ELISA e tutto il kit a  $-20^{\circ}\text{C}$ . È possibile rimuovere la piastra e conservarla a  $-20^{\circ}\text{C}$ , mentre i rimanenti reagenti del kit devono essere conservati tra  $2^{\circ}$  e  $8^{\circ}\text{C}$ . La scadenza del kit e dei reagenti è indicata sulle etichette.

La data di scadenza dei componenti del kit può essere garantita solo se questi sono conservati correttamente e, in caso di uso ripetuto di un componente, il reagente non è stato contaminato durante la prima manipolazione.

### **3 Preparazione Dei Reagenti**

#### **3.1 Tampone di lavaggio**

Se il tampone di lavaggio concentrato presenta cristalli in sospensione, riscaldare lievemente il tampone fino a ottenere la completa dissoluzione dei cristalli.

Versare l'intero contenuto (25 ml) del tampone di lavaggio concentrato in un cilindro graduato pulito da 500 ml. Portare il volume finale a 500 ml utilizzando acqua distillata o acqua deionizzata. Mescolare delicatamente per evitare la formazione di schiuma. Il pH della soluzione finale si deve regolare a 7,4.

Trasferire il prodotto in una bottiglia pulita e conservare a temperature comprese fra 2 °C e 25 °C. Il tampone di lavaggio è stabile per 30 giorni.

#### 4 Procedura del Test

- ~ Usare la piastra immediatamente dopo la rimozione dall'ambiente refrigerato a  $-20^{\circ}\text{C}$ !
  - ~ Non attendere la completa dissoluzione dei pellet prima di usare i campioni; la reazione di legame nelle strip degli étalon inizia immediatamente dopo l'aggiunta di acqua!
  - ~ Non cercare di dissolvere i pellet pipettando su e giù nei pozzetti; particelle del pellet possono aderire al puntale determinando una notevole variabilità dei risultati.
  - ~ Eseguire la fase di lavaggio con almeno  $400\ \mu\text{l}$  di tampone di lavaggio come indicato nel manuale o riempire completamente i pozzetti; in caso contrario non sarà possibile rimuovere tutti i residui del pellet adesi al bordo del pozzetto, determinando una notevole variabilità dei risultati.
  - ~ Rimuovere con cautela i fogli protettivi delle strip degli étalon per lasciare tutti i pellet liofilizzati nei pozzetti.
- a. Stabilire il numero di strip dei micropozzetti necessarie per analizzare la quantità desiderata di campioni più le **strip** per i blank e gli étalon (**colorate**). Tutti i campioni, gli étalon, il blank e i campioni di controllo opzionali devono essere processati in duplicato. Rimuovere dal supporto le **strip micropozzetti** non utilizzate e conservarle nella bustina metallica contenente la polvere essiccante, mantenendole a  $-20^{\circ}\text{C}$  e perfettamente sigillate. Mettere le strip contenenti la curva étalon nelle posizioni da A1/A2 a H1/H2 (vedere la Table 9).
  - b. Dispensare  $135\ \mu\text{l}$  di acqua distillata nei pozzetti dei campioni.
  - c. Dispensare acqua distillata a tutti i pozzetti per lo standard ed il bianco come indicato sull'etichetta degli standard strip (A1, A2 fino a H1, H2)

Table 9

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>
<b>A</b>	Standard 1 (10.00 ng/ml)	Standard 1 (10.00 ng/ml)	Campione 1	Campione 1
<b>B</b>	Standard 2 (5.00 ng/ml)	Standard 2 (5.00 ng/ml)	Campione 2	Campione 2
<b>C</b>	Standard 3 (2.50 ng/ml)	Standard 3 (2.50 ng/ml)	Campione 3	Campione 3
<b>D</b>	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Campione 4	Campione 4
<b>E</b>	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Campione 5	Campione 5
<b>F</b>	Standard 6 (0.32 ng/ml)	Standard 6 (0.32 ng/ml)	Campione 6	Campione 6
<b>G</b>	Standard 7 (0.16 ng/ml)	Standard 7 (0.16 ng/ml)	Campione 7	Campione 7
<b>H</b>	Blind Campione	Blind Campione	Campione 8	Campione 8

d. Dispensare 15 µl di ciascun **campione**, in duplicato, nei pozzetti di reazione e mescolare il contenuto.

Coprire con un **copripietra** e incubare a temperatura ambiente

e. (18-25 °C) per 2 ore utilizzando, se disponibile, un vortex a 100 rpm.

f. Rimuovere il **copripietra** e svuotare i pozzetti. Lavare 3 volte le strip micropozzetti utilizzando circa 400 µl di tampone di lavaggio per pozzetto, aspirando accuratamente il contenuto dei micropozzetti tra un lavaggio e l'altro. Evitare di scalfire la superficie dei micropozzetti.

g. Dopo l'ultimo lavaggio, asciugare le strip micropozzetti con un tampone o carta assorbente per rimuovere il tampone di lavaggio in eccesso. Utilizzare le strip subito dopo il lavaggio o sistemarle

capovolte su carta assorbente umida per non più di 15 min. Non lasciar asciugare i pozzetti.

- h. Pipettare 100 µl di **soluzione substrato TMB** in tutti i pozzetti, inclusi quelli del blank.
- i. Incubare le strip a temperatura ambiente (18-25° C) per circa 10 minuti. Evitare l'esposizione diretta a luci intense. **È necessario monitorare i valori O.D. a livello della piastra e interrompere la reazione del substrato prima che i pozzetti positivi cessino di essere appropriatamente registrabili.**  
Si raccomanda di aggiungere la soluzione di stop quando lo standard più elevato ha sviluppato un colore blu scuro.  
Alternativamente lo sviluppo del colore può essere monitorato con un lettore ELISA a 620 nm. La reazione del substrato deve essere bloccata non appena viene misurato un valore delle OD di 0.6 - 0.65.
- j. Interrompere la reazione enzimatica pipettando rapidamente 100 µl di **soluzione bloccante** in ciascun pozzetto, inclusi i pozzetti del blank. È importante che la soluzione bloccante si diffonda rapidamente e uniformemente attraverso i micropozzetti per inattivare completamente l'enzima. I risultati devono essere letti immediatamente dopo l'aggiunta della soluzione bloccante o entro 1 ora se le strip sono conservate in un luogo buio a 2-8° C.
- k. Leggere l'assorbanza di ciascun micropozzetto su uno spettrofotometro che utilizza 450 nm come lunghezza d'onda primaria (620 nm come lunghezza d'onda di riferimento alternativa; valori da 610 nm a 650 nm sono accettabili). Azzerare il lettore della piastra secondo le istruzioni del produttore e utilizzando i pozzetti del blank. Determinare l'assorbanza sia dei campioni, sia degli étalon di human sCD40L.