

READ BEFORE OPENING

- Vials may contain small quantities of material, hence ensure that they are centrifuged prior to opening.
- This set of reagents is intended for use by persons experienced in the use of immunoassays. It is not suitable for use by inexperienced personnel.
- A sample protocol is included but please note that the protocol provided is a guideline. The type of substrate as well as all other reagents not included in the module set may influence test performance.

WORKING PROTOCOL FOR THE sCD30 MODULE SET BMS240MST

1) Reagents provided (for 10 ELISA plates):

- 5.5 ml coating antibody (100 µg/ ml)
- 1 vial sCD30 standard protein lyophilized
- 55 µl HRP-conjugate
- 100 ml Sample Diluent

2) Buffers and further materials needed:

a) Phosphate buffered saline (PBS)

NaCl	8.00 g
KCl	0.20 g
Na ₂ HPO ₄ x 12 H ₂ O	2.85 g
KH ₂ PO ₄	0.20 g

Dissolve the salts in distilled water and adjust to 1 litre.

b) Assay Buffer:

Bovine Serum Albumin (BSA)	5 g
Tween 20	0.5 ml
PBS	adjust to 1 litre.

Dissolve ingredients in approx. 500ml PBS, then adjust to 1 litre with PBS.

- c) Wash Buffer:
Add 0.5 ml Tween 20 to 1 litre of PBS and mix well.
- d) Microwell plate (Maxi sorb)
- e) Substrate Solution: 1:2 mixture of H₂O₂ and Tetramethylbenzidine (KPL Gaithersburg, Maryland)
- f) Stop Solution: 4N Sulfuric Acid (2 ml conc. (36N) Sulfuric Acid + 16 ml H₂O).

3) Storage condition:

Store the reagents of the module set at -20°C. Immediately after use reagents should be returned to -20°C storage. Avoid several freeze-thaw cycles. Aliquot reagents for use at different time points. Expiry of the reagents is stated on labels.

4) Preparation of reagents:

Please note: Centrifuge vials before opening to collect contents.

- a) Preparation of the microwell Plate:

Coating:

The final antibody concentration is 5 µg/ ml; 100 µl of the coating solution are added to each well. Dilute the coating antibody as following for one microtiter plate:

10.45 ml	PBS
550.0 µl	coating antibody (100 µg/ ml)
<hr/>	
11.0 ml	coating solution

Immediately after coating, seal the plate with a plate cover and transfer to 2-8°C, allowing the binding process to take place over night.

Aspirate the contents of the wells and wash once with about 300 µl of Wash Buffer according the Washing procedure described in the test protocol below.

Blocking:

Add 250 µl of Assay Buffer to each well and allow the binding reaction to take place for two hours at room temperature (alternatively the plate may be blocked over night at 2-8°C).

Wash the plate twice (see below) immediately before the samples are added to the wells. The blocked plates can be stored at 2-8°C up to one week.

Fixing:

If you want to store the coated plates for a longer period of time, just aspirate the blocking solution and proceed by adding 150 µl Fixing solution (PBS, 15% Sucrose) to each well. Incubate 1 h at room temperature, aspirate and dry plates for over night at 28°C. When sealed with desiccant, the plates can be stored at 2-8°C for at least 2 months.

b) Preparation of Standard:

The final concentration of the sCD30 standard protein is 200 ng/ ml. Reconstitute the lyophilized protein with distilled water as indicated on the label of the standard vial. Dilute the stock material as following for one standard curve:

25 µl	Standard Protein (2000 ng/ ml)
225 µl	Assay Buffer
<hr/>	
250.0 µl	Standard Protein (200 ng/ ml)

Store the reconstituted Standard aliquoted at -20°C. Aliquoted Standard is stable for 3 months at -20°C

c) Preparation of HRP-Conjugate:

The HRP-Conjugate must be diluted 1:1000 with Assay Buffer before use. Dilute the stock material as following for one microwell plate:

5.5 μ l	HRP-Conjugate
5494.5 μ l	Assay Buffer
<hr/>	
5500.0 μ l	HRP-Conjugate

The reagents are now ready to be used in the test according to the test protocol below.

TEST PROTOCOL

- a. Add 100 μ l of **Sample Diluent**, in duplicate, to all standard wells. Prepare standard dilutions by pipetting 100 μ l of diluted **sCD30 Standard**, in duplicate, into well A1 and A2 (see Figure 1 and 2). Mix the contents of wells A1 and A2 by repeated aspiration and ejection, and transfer 100 μ l to well B1 and B2, respectively. Take care not to scratch the inner surface of the microwells. Continue this procedure five times, creating two rows of sCD30 Standard dilutions ranging from 100 to 1.6 ng/ ml. Discard 100 μ l of the contents from the last microwells used (G1, G2).

Figure 1. Preparation of sCD30 standard dilutions:

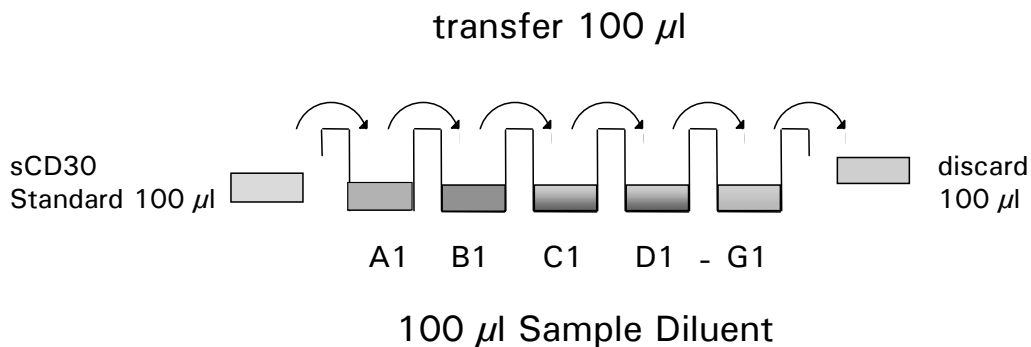


Figure 2. Diagram depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (100 ng/ml)	Standard 1 (100 ng/ml)	Sample 1	Sample 1
B	Standard 2 (50 ng/ml)	Standard 2 (50 ng/ml)	Sample 2	Sample 2
C	Standard 3 (25 ng/ml)	Standard 3 (25 ng/ml)	Sample 3	Sample 3
D	Standard 4 (12.5 ng/ml)	Standard 4 (12.5 ng/ml)	Sample 4	Sample 4
E	Standard 5 (6.3 ng/ml)	Standard 5 (6.3 ng/ml)	Sample 5	Sample 5
F	Standard 6 (3.2 ng/ml)	Standard 6 (3.2 ng/ml)	Sample 6	Sample 6
G	Standard 7 (1.6 ng/ml)	Standard 7 (1.6 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

- b. Add 100 μ l of **Sample Diluent**, in duplicate, to the blank wells.
- c. Add 75 μ l of **Sample Diluent** to all wells designated for samples.
- d. Add 25 μ l of each **Sample**, in duplicate, to the designated wells and mix the contents.
- e. Prepare **HRP-Conjugate** (Refer to preparation of reagents).
- f. Add 50 μ l of diluted **HRP-Conjugate** to all wells, including the blank wells.
- g. Cover with a **Plate Cover** and incubate at room temperature (18°C to 25°C) for 3 hours, if available on a rotator set at 100 rpm.
- h. Prepare TMB Substrate Solution a few minutes prior to use.

- i. Remove plate cover and empty wells. Wash the microwell strips 4 times with approximately 400 μ l Wash Buffer per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.
After the last wash, empty wells and 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 not longer than 15 minutes. Do not allow wells to dry.
- j. Pipette 100 μ l of mixed **TMB Substrate Solution** to all wells, including the blank wells.
- k. Incubate the microwell strips at room temperature (18° to 25°C) for about 10 minutes, if available on a rotator set at 100 rpm. 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 an OD of 0.6 – 0.65 is reached.
- l. Stop the enzyme reaction by quickly pipetting 100 μ l of **4N Sulfuric Acid** into each well, including the blank wells. It is important that the Sulfuric Acid is spread quickly and uniformly throughout the microwells to completely inactivate the enzyme. Results must be read immediately after the Sulfuric Acid is added or within one hour if the microwell strips are stored at 2 - 8°C in the dark.
- m. 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 sCD30 standards.

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 sCD30 concentration on the abscissa. Draw a best fit curve through the points of the graph.
- To determine the concentration of circulating sCD30 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 sCD30 concentration.
- **For samples which have been diluted according to the instructions given in this manual 1 : 4 the concentration read from the standard curve must be multiplied by the dilution factor (x 4).**

Note: Calculation of samples with an O.D. exceeding 2.0 may result in incorrect low sCD30 levels. Such samples require further dilution in order to precisely quantitate the actual sCD30 level.

- It is suggested that each testing facility establishes a control sample of known sCD30 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 basic understanding of immunoassay development and technical experience in ELISA performance are prerequisite for the successful use of this module set.

The protocol provided is just a guideline. The type of substrate as well as all other reagents not included in the module set may influence the test characteristics.

GENERAL INFORMATION

Summary

The CD30 (Ki-1) molecule was identified by a monoclonal antibody which was originally found to react with an epitope present in Hodgkin's and Reed-Sternberg cells in Hodgkin's disease (23). Later, the Ki-1 antigen was found to be consistently expressed by a subgroup of diffuse large-cell lymphomas that were called Ki-1 positive (Ki-1⁺) anaplastic large-cell lymphomas (ALCL) (26).

Characterization of the CD30 antigen has shown it to be in its mature form a transmembrane protein of about 120kDa (12, 22) elaborated from an 84kD cytoplasmic precursor primarily through glycosylation (22). The cloning of the CD30 gene has allowed the identification of a cDNA with an open reading frame predicting a 595 amino acid polypeptide (7). The extracellular domain of CD30, comprising 365 residues, has proved to be homologous to that of the TNF-receptor superfamily (1). The CD30 gene is localized at chromosome 1q36 (11), closely linked to other members of the TNF receptor superfamily comprising TNF-receptors, nerve growth factor, CD40, APO-1/Fas, CD27, OX40 and the neurotrophin receptor. The CD30 ligand (CD30L) has been identified, showing significant homology to TNF α , TNF β , FasL, CD40L, CD27L and 4-1BBL (2, 9, 25). CD30L is expressed on activated T-cells (24). Interactions of the cytokine receptor CD30 with its ligand induces pleiotropic biologic effects, such as differentiation, activation, proliferation and cell death (14). In CD30⁺ ALCL cell lines binding of CD30L induces apoptotic cell death (14). CD30 furthermore seems to be involved in the control of the CD40/CD40L signal, T-cell proliferation and B-cell maturation induced by T-cell cytokines (6). Thus, CD30 seems to transmit information that is essential for the immune response.

CD30 expression is strictly dependent on activation and proliferation of T- and B-cells (27). In pathological conditions, CD30 positivity is regarded as a peculiar attribute of Hodgkin's and Reed-Sternberg cells (4).

There is growing evidence for a potential role of the CD30 molecule in clinical use and therapy (8). An 85kDa soluble form of the CD30

molecule (sCD30) has been shown to be released by CD30⁺ cell in vitro and in vivo (16).

It is probably derived from the 120kDa membrane bound molecule by proteolytic cleavage (15). Serum sCD30 detection can be regarded as a marker of the amount of CD30⁺ cells present in the body.

Increased serum levels of sCD30 have been reported for patients with CD30⁺ ALCL (19) and CD30⁺ embryonal carcinoma (18) of the testis and were found to correlate with the clinical phase of the disease, i.e. presentation complete remission (CR), relapse. Elevated serum values of sCD30 are shown in the majority of patients with Hodgkin's Disease (20, 21) which again correlate with the presence of B symptoms and with the stage of the disease, i.e. tumor burden.

While elevations of the soluble CD30 in serum of patients affected by infections diseases usually are not detected, infectious mononucleosis is a notable exception (28). Serum levels of sCD30 are also increased in most patients with HBsAg-positive chronic hepatitis and signs of active HBV replication, thus there is association of the raised sCD30 levels with the active phase of the illness (10). Abnormal soluble CD30 serum accumulation has been reported in Omenn's syndrome, a severe immunodeficiency (5). High elevations of sCD30 levels are found in patients of systemic lupus erythematosus which correlate with disease activity (3), in patients with the autoimmune liver disease primary biliary cirrhosis (17) and in patients with rheumatoid arthritis (13).

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Specificity

The interference of circulating factors of the immune systems was evaluated by spiking these proteins at physiologically relevant concentrations into a sCD30 positive serum. There was no detectable cross reactivity.

Expected Values

A panel of 32 sera from apparently healthy blood donors (male and female) was tested for sCD30. The detected sCD30 levels ranged between 17.5 and 130.7 ng/ml with a mean level of 38.7 ng/ml and a standard deviation of ± 28.0 ng/ml.

ORDERING INFORMATION

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