Version 13-07/24

User's Manual

Human sE-Selectin ELISA Kit

REF **DEIA4564**

Σ **96T**

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This product is for research use only and is not intended for diagnostic use.

For illustrative purposes only. To perform the assay the instructions for use provided with the kit have to be used.

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PRODUCT INFORMATION

Intended Use

The human sE-selectin ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human sE-selectin. The human sE-selectin ELISA is for research use only. Not for diagnostic or therapeutic procedures.

General Description

Endothelial Leukocyte Adhesion Molecule-1 (ELAM-1, E-selectin) belongs to the selectin family of adhesion molecules. Together with LECAM-1 (L-selectin) and GMP-140 (P-selectin), Eselectin mediates the initial interactions of leukocytes and platelets with endothelial cells. Molecular structure: The extracellular part of all selectins consists of an aminoterminal c-type lectin domain which specifically binds to carbohydrate ligands. This is followed by an EGF-like domain, and, in the case of E-selectin, by 6 short consensus repeats. The transmembrane portion of the molecule is followed by a short cytoplasmic tail. Selectins guide non-activated polymorphonuclear cells to the areas of inflammation in creating first, loose contacts with the endothelial layer. The potential binding partner of E-selectin contains sialyl LewisX oligosaccharide. Other suitable ligands for the lectin domain of Eselectin are sialylated, fucosylated lactosaminoglycans. Together with GMP-140, E-selectin is expressed on cytokine-activated endothelial cells, and contributes to the adhesion of still resting leukocytes to the endothelium. This initial binding event is an essential prerequisite for the activation of the immune cells via different inflammatory mediators. In contrast to GMP-140, E-selectin is maximally expressed 2-4 hours after cell activation. Within the next 24-48 hours E-selectin is again eliminated from the cytoplasmic membrane by shedding into the circulation. The circulating form or soluble (sE-selectin) of this selectin exerts chemotactical signals on neutrophils and additionally activates the 2-integrins - sE-selectin assists in preparing the migration capacity of these cells.

Principles of Testing

1. An anti-human sE-selectin coating antibody is adsorbed onto microwells.
2. Human sE-selectin present in the sample or standard binds to antibodies adsorbed to the microwells. A HRP-conjugated anti-human sE-selectin antibody is added and binds to human sE-selectin captured by the first antibody.
3. Following incubation unbound HRP-conjugated anti-human sE-selectin is removed during a wash step, and substrate solution reactive with HRP is added to the wells.
4. A coloured product is formed in proportion to the amount of human sE-selectin present in the sample or standard. The reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from 6 human sE-selectin standard dilutions and human sE-selectin concentration determined.

Reagents And Materials Provided

1. 1 aluminium pouch with a **Antibody Coated Microtiter Strips** with monoclonal antibody to human sE-selectin

2. 1 vial (150 µl) **HRP-Conjugate** anti-human sE-selectin monoclonal antibody
3. 2 vials human sE-selectin **Standard** lyophilized, 100 ng/ml upon reconstitution
4. 1 vial **Control high**, lyophilized
5. 1 vial **Control low**, lyophilized
6. 1 vial (12 ml) **Sample Diluent**
7. 1 vial (5 ml) **Assay Buffer Concentrate** 20× (PBS with 1% Tween 20 and 10% BSA)
8. 1 bottle (50 ml) **Wash Buffer Concentrate** 20× (PBS with 1% Tween 20)
9. 1 vial (15 ml) **Substrate Solution** (tetramethyl-benzidine)
10. 1 vial (15 ml) **Stop Solution** (1M Phosphoric acid)
11. 2 **Adhesive Films**

Materials Required But Not Supplied

1. 5 ml and 10 ml graduated pipettes
2. 5 µl to 1000 µl adjustable single channel micropipettes with disposable tips
3. 50 µl to 300 µl adjustable multichannel micropipette with disposable tips
4. Multichannel micropipette reservoir
5. Beakers, flasks, cylinders necessary for preparation of reagents
6. Device for delivery of wash solution (multichannel wash bottle or automatic wash system)
7. Microwell strip reader capable of reading at 450 nm (620 nm as optional reference wave length)
8. Glass-distilled or deionized water
9. Statistical calculator with program to perform regression analysis

Storage

Store the complete kit at 2-8°C. Under these conditions, the kit is stable until the expiration date (see label on the box).

Specimen Collection And Preparation

Cell culture supernatant, serum and plasma (citrate and heparin) were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum or plasma from the clot or 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 should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human sE-selectin. If samples are to be run within 24 hours, they may be stored at 2-8°C. Avoid repeated freeze-thaw cycles. Prior to assay, the frozen sample should be brought to room temperature slowly and mixed gently.

Sample Stability

a. Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human sE-

selectin levels determined. There was no significant loss of human sE-selectin immunoreactivity detected by freezing and thawing.

b. 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 sE-selectin level determined after 24 h. There was no significant loss of human sE-selectin immunoreactivity detected during storage under above conditions.

Reagent Preparation

Buffer concentrates should be brought to room temperature and should be diluted before starting the test procedure. If crystals have formed in the Buffer Concentrates, warm them gently until they have completely dissolved.

1. **Wash Buffer (1×)**

Pour entire contents (50 ml) of the **Wash Buffer Concentrate** (20×) into a clean 1000 ml graduated cylinder. Bring to final volume of 1000 ml with glass-distilled or deionized water. Mix gently to avoid foaming. Transfer to a clean wash bottle and store at 2-25°C. Please note that Wash Buffer (1×) is stable for 30 days. Wash Buffer (1×) may also be prepared as needed according to the following table:

Number of Strips	Wash Buffer Concentrate 20× (ml)	Distilled Water (ml)
1-6	25	475
1-12	50	950

2. **Assay Buffer** (1×)

Pour the entire contents (5 ml) of the **Assay Buffer Concentrate** (20×) into a clean 100 ml graduated cylinder. Bring to final volume of 100 ml with distilled water. Mix gently to avoid foaming. Store at 2-8°C. Please note that the Assay Buffer (1x) is stable for 30 days. Assay Buffer (1×) may also be prepared as needed according to the following table:

Number of Strips	Assay Buffer Concentrate 20× (ml)	Distilled Water (ml)
1-6	2.5	47.5
1-12	5.0	95.0

3. **HRP-Conjugate**

Please note that the HRP-Conjugate should be used within 30 minutes after dilution. Make a 1:100 dilution of the concentrated **HRP-Conjugate** solution with Assay Buffer (1×) in a clean plastic tube as needed according to the following table:

Number of Strips	HRP-Conjugate (ml)	Assay Buffer 1× (ml)
1-6	0.03	2.97
1-12	0.06	5.94

4. **Human sE-selectin Standard**

Reconstitute **human sE-selectin standard** by addition of distilled water. Reconstitution volume is stated in

Quality Control Sheet. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 100 ng/ml). Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions. The standard has to be used immediately after reconstitution and cannot be stored.

5. **External Standard Dilution**

Label 6 tubes, one for each standard point.

S1, S2, S3, S4, S5, S6

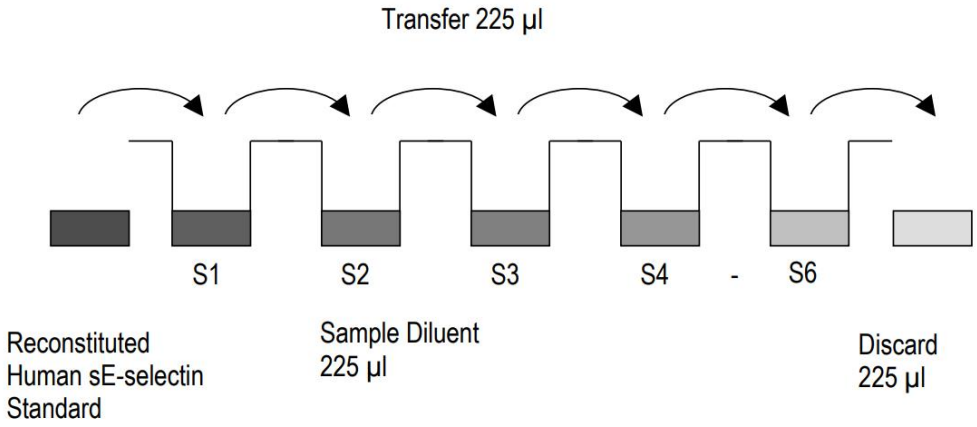
Then prepare 1:2 serial dilutions for the standard curve as follows:

Pipette 225 µl of Sample Diluent into each tube.

Pipette 225 µl of reconstituted standard (concentration = 100 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 50 ng/ml).

Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer. Repeat serial dilutions 4 more times thus creating the points of the standard curve (see Figure).

Sample Diluent serves as blank.



6. **Controls**

Reconstitute lyophilized controls by addition of distilled water (10-30 minutes). Swirl or mix gently to ensure complete and homogeneous solubilization. Further treat the controls like your samples in the assay. For control range please refer to certificate of analysis. Store reconstituted controls aliquoted at -20°C. Avoid repeated freeze and thaw cycles.

Assay Procedure

1. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. 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 2-8°C sealed tightly.
2. Wash the microwell strips twice with approximately 400 µl **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 step, empty wells and tap microwell strips on absorbent pad or paper towel to remove excess Wash Buffer. Use the microwell strips immediately after washing. Alternatively microwell strips can be placed upside down on a wet absorbent paper for not longer than 15 minutes. **Do not allow wells to dry.**

3. **Standard dilution on the microwell plate** (Alternatively the standard dilution can be prepared in tubes): Add 100 µl of Sample Diluent in duplicate to all **standard wells**. Pipette 100 µl of prepared **standard** (see **Reagents Preparation of 5**, concentration = 100.0 ng/ml) in duplicate into well A1 and A2 (see Table)Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 50.0 ng/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure.) Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human sE-selectin standard dilutions ranging from 50.0 to 1.6 ng/ml. Discard 100 µl of the contents from the last microwells (F1, F2) used.

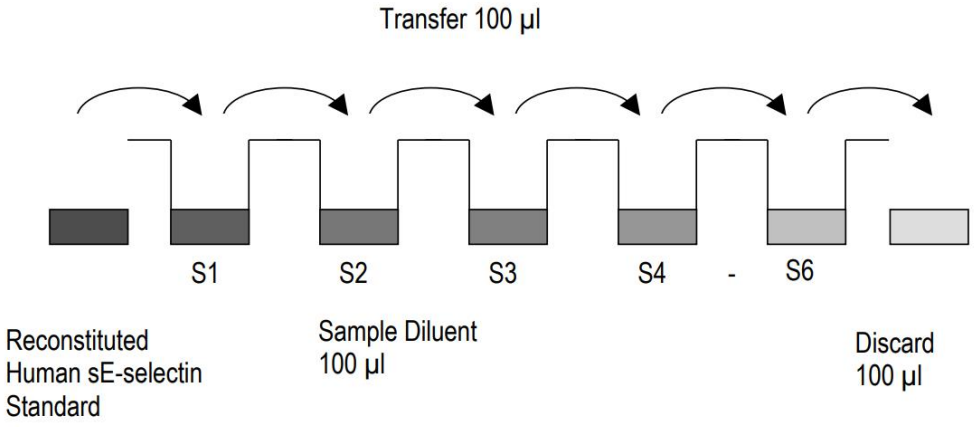


Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips:

	1	2	3	4
A	Standard 1 (50.0 ng/ml)	Standard 1 (50.0 ng/ml)	Sample 2	Sample 2
B	Standard 2 (25.0 ng/ml)	Standard 2 (25.0 ng/ml)	Sample 3	Sample 3
C	Standard 3 (12.5 ng/ml)	Standard 3 (12.5 ng/ml)	Sample 4	Sample 4
D	Standard 4 (6.3 ng/ml)	Standard 4 (6.3 ng/ml)	Sample 5	Sample 5
E	Standard 5 (3.1 ng/ml)	Standard 5 (3.1 ng/ml)	Sample 6	Sample 6
F	Standard 6 (1.6 ng/ml)	Standard 6 (1.6 ng/ml)	Sample 7	Sample 7
G	Blank	Blank	Sample 8	Sample 8
H	Sample 1	Sample 1	Sample 9	Sample 9

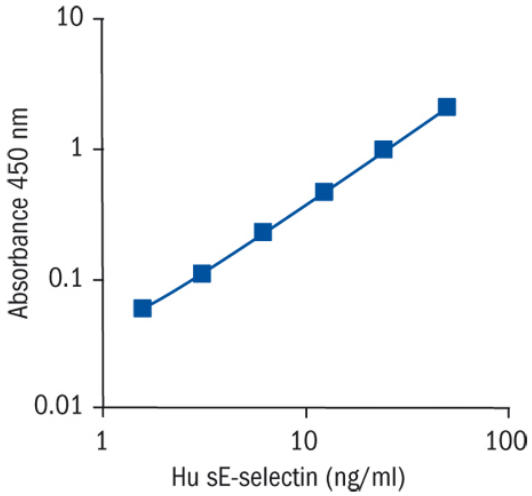
4. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
5. Add 80 µl of **Sample Diluent** to the **sample wells**.
6. Add 20 µl of each **sample** in duplicate to the **sample wells**.
7. Prepare **HRP-Conjugate** (see **Reagents Preparation 3**).
8. Add 50 µl of **HRP-Conjugate** to all wells.
9. Cover with an adhesive film and incubate at room temperature (18-25°C) for 2 hours, if available on a microplate shaker.
10. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to point 2 of the test Procedure. Proceed immediately to the next step.

11. Pipette 100 µl of **TMB Substrate Solution** to all wells.
12. Incubate the microwell strips at room temperature (18-25°C) for about 10 min. Avoid direct exposure to intense light. **The colour development on the plate should be monitored and the substrate reaction stopped (see next point of this Procedure) before positive wells are no longer properly recordable. Determination of the ideal time period for colour development has to be done individually for each assay.** 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 Standard 1 has reached an OD of 0.9-0.95.
13. Stop the enzyme reaction by quickly pipetting 100 µl of **Stop Solution** into each well. 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.
14. 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 standards.

Calculation

1. Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.
2. Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human sE-selectin concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).
3. To determine the concentration of circulating human sE-selectin 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 sE-selectin concentration.
4. **If instructions in this protocol have been followed samples have been diluted 1:5 (20 µl sample + 80 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (×5).**
5. **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human sE-selectin levels. Such samples require further external predilution according to expected human sE-selectin values with Sample Diluent in order to precisely quantitate the actual human sE-selectin level.**
6. It is suggested that each testing facility establishes a control sample of known human sE-selectin 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.

Typical Standard Curve



Reference Values

Panels of 40 serum as well as citrate and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human sE-selectin. For detected human sE-selectin levels see Table.

Sample Matrix	Number of Samples Evaluated	Range (ng/ml)	Mean (ng/ml)	Standard Deviation (ng/ml)
Serum	40	21.0 - 186.0	66.5	34.8
Plasma (Citrate)	40	17.5 - 88.1	50.5	20.7
Plasma (Heparin)	40	18.1 - 105.3	67.2	27.8

Detection Range

1.6-50 ng/ml

Detection Limit

0.3 ng/ml

Sensitivity

The limit of detection of human sE-selectin 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 0.3 ng/ml (mean of 6 independent assays).

Specificity

The assay detects both natural and recombinant human sE-selectin. The interference of circulating factors of

the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into a human sE-selectin positive serum. There was no crossreactivity detected, namely not with IL-8, sICAM-1, sTNF-R, TNFβ, CD8, ÌL-2R, IL-6, sL-selectin and sP-selectin.

Linearity

4 serum samples with different levels of human sE-selectin were analysed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 89% to 106% with an overall recovery of 95% (see Table).

Sample	Dilution	Expected Human sE-selectin Concentration (ng/ml)	Observed Human sE-selectin Concentration (ng/ml)	Recovery of Expected Human sE-selectin Concentration (%)
1	1:5	--	52.8	--
	1:10	26.4	25.3	96
	1:20	12.6	11.6	92
	1:40	5.8	6.1	105
2	1:5	--	36.5	--
	1:10	18.2	18.5	102
	1:20	9.3	8.3	89
	1:40	4.1	3.7	89
3	1:5	--	51.1	--
	1:10	25.5	23.4	92
	1:20	11.7	10.6	91
	1:40	5.3	5.0	94
4	1:5	--	48.9	--
	1:10	24.5	22.6	92
	1:20	11.3	10.3	92
	1:40	5.2	5.5	106

Recovery

The spiking recovery was evaluated by spiking 3 levels of human sE-selectin into serum samples. Recoveries were determined in 3 independent experiments with 4 replicates each. The amount of endogenous human sE-selectin in unspiked serum was subtracted from the spike values. The recovery ranged from 71% to 95% with an overall mean recovery of 86%.

Reproducibility

1. **Intra-assay**

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human sE-selectin. 2 standard curves were run on each plate. Data below show the mean human sE-selectin concentration and the coefficient of variation for each sample (see Table). The calculated overall intra-assay coefficient of variation was 5.4%.

The mean human sE-selectin concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human sE-selectin Concentration (ng/ml)	Coefficient of Variation (%)
1	1	32.3	5.1
	2	33.7	4.6
	3	38.3	5.6
2	1	35.8	3.6
	2	35.4	6.1
	3	38.7	5.8
3	1	37.1	3.6
	2	36.2	7.2
	3	39.8	3.0
4	1	30.3	4.9
	2	28.0	2.2
	3	30.0	2.7
5	1	34.4	3.3
	2	40.1	3.4
	3	35.7	4.7
6	1	12.3	8.5
	2	12.1	7.7
	3	12.3	4.7
7	1	23.7	4.7
	2	22.2	8.7
	3	24.5	6.9
8	1	19.6	8.0
	2	17.2	6.1
	3	22.9	7.6

2. **Inter-assay**

Assay to assay reproducibility within one laboratory was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of human sE-selectin. 2 standard curves were run on each plate. Data below show the mean human sE-selectin concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table). The calculated overall inter-assay coefficient of variation was 6.0%.

The mean human sE-selectin concentration and the coefficient of variation of each sample

Sample	Mean Human sE-selectin Concentration (ng/ml)	Coefficient of Variation (%)
1	34.8	9.1
2	36.6	4.9
3	37.7	4.9
4	29.4	4.3
5	36.7	8.1
6	12.3	0.9
7	23.5	5.1
8	19.9	14.4

Precautions

1. 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 statement(s) for specific advice.

2. Reagents are intended for research use only and are not for use in diagnostic or therapeutic procedures.
3. Do not mix or substitute reagents with those from other lots or other sources.
4. Do not use kit reagents beyond expiration date on label.
5. Do not expose kit reagents to strong light during storage or incubation.
6. Do not pipette by mouth.
7. Do not eat or smoke in areas where kit reagents or samples are handled.
8. Avoid contact of skin or mucous membranes with kit reagents or specimens.
9. Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
10. Avoid contact of substrate solution with oxidizing agents and metal.
11. Avoid splashing or generation of aerosols.
12. 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.
13. Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
14. Exposure to acid inactivates the conjugate.
15. Glass-distilled water or deionized water must be used for reagent preparation.
16. Substrate solution must be at room temperature prior to use.
17. 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.
18. 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.

Limitations

1. Since exact conditions may vary from assay to assay, a standard curve must be established for every run.
2. Bacterial or fungal contamination of either screen samples or reagents or crosscontamination between reagents may cause erroneous results.
3. Disposable pipette tips, flasks or glassware are preferred, reusable glassware must be washed and thoroughly rinsed of all detergents before use.
4. 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.
5. 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.

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