Version 21-08/24

User's Manual

PSGL-1 ELISA Kit

REF **DEIA6210**

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RUO

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.

Creative Diagnostics

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PRODUCT INFORMATION

Intended Use

The human PSGL-1 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human PSGL-1. The human PSGL-1 ELISA is for research use only. Not for diagnostic or therapeutic procedures.

General Description

The family of selectins consists of three structurally and functionally related molecules. L-selectin is constitutively expressed on neutrophils, P-selectin is found on platelets and is stored in Weibel-Palade bodies from where it is transported to the cell surface upon endothelial activation. E-selectin is expressed on endothelial cells. Due to a common structural element, the aminoterminal lectin-like domain, the selectins are able to bind to carbohydrate ligands. Different putative ligand structures have been identified for which the selectins show high affinity. These structures include oligosaccharides, phophorylated saccharides, sulfopolysaccharides and lipids. It was shown that glycoproteins represent the biological relevant ligands for selectins. While the ligands for E- and L-selectins with primary binding activity have not been identified so far, the functionally most important ligand for P-selectin has been identified. The mucin-like glycoprotein PSGL-1 (P-selectin Glycoprotein Ligand-1) has been cloned and sequenced.

PSGL-1 has been shown to be a transmembrane protein which forms homodimers via disulfide bridges of two 120 kDa chains.

PSGL-1 is expressed on cells of myeloid, lymphoid and dendritic lineage. The binding of P-selectin is regulated by different degrees and forms of glycosylation. An interaction of L-selectin with PSGL-1 in the process of neutrophil aggregation has been shown. However, PSGL-1 does not seem to be the primary ligand for L-selectin.

Presently it is not known which cells, apart from leukocytes, express PSGL-1 and what role PSGL-1 plays on these cells. The metastic potential of the majority of cells which bind to P-selectin is however in close correlation with the functional expression of PSGL-1 on these cells.

The regulation of PSGL-1 is not yet well described. Glycosyltransferases sure play an important role in activation. The deactivation of PSGL-1 is so far unclear. The cleavage of the protein from the cell surface is one mechanism involved in the deactivation process.

Following this shedding, a soluble form of PSGL-1 is detectable in the circulation. This soluble isoform of PSGL-1 is still capable of binding to P-selectin, thus representing a competitor for cellular PSGL-1 through which regulation in many physiological and pathological processes can take place.

Principles of Testing

An anti-human PSGL-1 monoclonal coating antibody is adsorbed onto microwells.

Human PSGL-1 present in the sample or standard binds to antibodies adsorbed to the microwells; a biotin-conjugated monoclonal antihuman PSGL-1 antibody is added and binds to human PSGL-1 captured by the first antibody.

Following incubation unbound biotin-conjugated anti-human PSGL-1 is removed during a wash step.

Streptavidin-HRP is added and binds to the biotin-conjugated anti-human PSGL-1 antibody.

Following incubation unbound Streptavidin-HRP 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 PSGL-1 present in the sample. The reaction is terminated by addition of acid and absorbance is measured at 450nm. A standard curve is prepared from six human PSGL-1 standard dilutions and human PSGL-1 sample concentration determined.

Reagents And Materials Provided

1. 1 aluminium pouch with a Microwell Plate coated with monoclonal antibody to human PSGL-1
2. 1 vial (70 µl) Biotin-Conjugate anti-human PSGL-1 monoclonal antibody
3. 1 vial (150 µl) Streptavidin-HRP
4. 2 vials human PSGL-1 Standard lyophilized, 100 U/ml upon reconstitution
5. 1 vial (50 ml) Sample Diluent
6. 1 vial (5 ml) Assay Buffer Concentrate 20x (PBS with 1% Tween 20, 10% BSA)
7. 1 bottle (50 ml) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)
8. 1 vial (15 ml) Substrate Solution (tetramethyl-benzidine)
9. 1 vial (15 ml) Stop Solution (1M Phosphoric acid)
10. 4 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 linear regression analysis.

Storage

Store kit reagents between 2° and 8°C. Immediately after use remaining reagents should be returned to cold storage (2 - 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.

Specimen Collection And Preparation

Cell culture supernatant and serum were tested with this assay. Other biological samples might be suitable for use in the assay. Remove serum from the clot as soon as possible after clotting.

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 PSGL-1. 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

Freeze-Thaw Stability

Aliquots of serum samples (spiked or unspiked) were stored at -20°C and thawed 5 times, and the human PSGL-1 levels determined. There was no significant loss of human PSGL-1 immunoreactivity detected by freezing and thawing.

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 PSGL-1 level determined after 24 h. There was no significant loss of human PSGL-1 immunoreactivity detected during storage under above conditions.

Reagent Preparation

Buffer Concentratesshould 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 (1x)**

Pour entire contents (50 ml) of the Wash Buffer Concentrate (20x) 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(1x) is stable for 30 days.

Wash Buffer (1x) may also be prepared as needed according to the following table:

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

2. **Assay Buffer (1x)**

Pour the entire contents (5 ml) of the Assay Buffer Concentrate (20x) 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 (1x) may also be prepared as needed according to the following table:

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

3. **Biotin-Conjugate**

Please note that the Biotin-Conjugate should be used within 30 minutes after dilution.

Make a 1:100 dilution of the concentrated Biotin-Conjugatesolution with Assay Buffer (1x) in a clean plastic tube as needed according to the following table:

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

4. **Streptavidin-HRP**

Please note that the Streptavidin-HRP should be used within 30 minutes after dilution.

Make a 1:200 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer(1x) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin-HRP (ml)	Assay Buffer (1x) (ml)
1 - 6	0.03	5.97
1 - 12	0.06	11.94

5. **Human PSGL-1 Standard**

Reconstitute human PSGL-1 standardby addition of distilled water.

Reconstitution volume is stated on the label of the standard vial. Allow the reconstituted standard to sit for 10-30 minutes. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 100 U/ml).

After usage remaining standard cannot be stored and has to be discarded.

Standard dilutions can be prepared directly on the microwell plate or alternatively in tubes.

5. 1 **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.

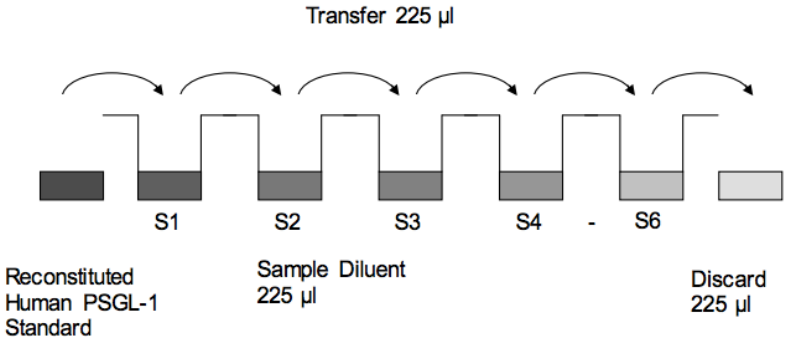
Pipette 225 µl of reconstituted standard (concentration = 100 U/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 50 U/ml).

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 1).

Sample Diluent serves as blank.

Figure 1



Assay Procedure

a. Predilute your samples before starting with the test procedure.

Dilute serum and plasma samples 1:20 with Sample Diluent according to the following scheme: 15 µl sample + 285 µl Sample Diluent

For cell culture supernatants optimal dilutions have to be determined.

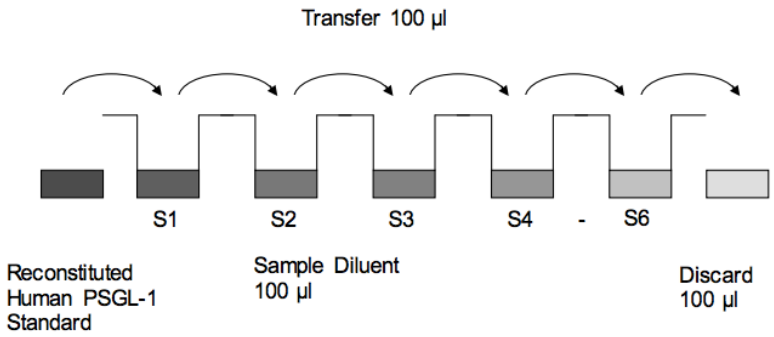
b. Determine the number of microwell strips required to test the desired number of samples plus appropriate number of wells needed for running blanks and standards. Each sample, standard, blank and optional control sample should be assayed in duplicate. Remove extra microwell strips from holder and store in foil bag with the desiccant provided at 2-8°C sealed tightly.

c. 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.

d. Standard dilution on the microwell plate (Alternatively thestandard dilution can be prepared in tubes - see External Standard Dilution):

Add 100 µl of Sample Diluent in duplicate to all standard wells. Pipette 100 µl of prepared standard (see Preparation of Standard, concentration = 100 U/ml) in duplicate into well A1 and A2 (see Table 1). Mix the contents of wells A1 and A2 by repeated aspiration and ejection (concentration of standard 1, S1 = 50 U/ml), and transfer 100 µl to wells B1 and B2, respectively (see Figure 2). Take care not to scratch the inner surface of the microwells. Continue this procedure 4 times, creating two rows of human PSGL-1 standard dilutions ranging from 50.00 to 1.56 U/ml. Discard 100 µl of the contents from the last microwells (F1, F2) used.

Figure 2



In case of an external standard dilution (see 5.1 **External Standard Dilution**), pipette 100 µl of these standard dilutions (S1 - S6) in the standard wells according to Table 1.

Table depicting an example of the arrangement of blanks, standards and samples in the microwell strips (Table 1):

Table 1

	1	2	3	4
A	Standard 1 (50.00 U/ml)	Standard 1 (50.00 U/ml)	Sample 2	Sample 2
B	Standard 2 (25.00 U/ml)	Standard 2 (25.00 U/ml)	Sample 3	Sample 3
C	Standard 3 (12.50 U/ml)	Standard 3 (12.50 U/ml)	Sample 4	Sample 4
D	Standard 4 (6.25 U/ml)	Standard 4 (6.25 U/ml)	Sample 5	Sample 5
E	Standard 5 (3.13 U/ml)	Standard 5 (3.13 U/ml)	Sample 6	Sample 6
F	Standard 6 (1.56 U/ml)	Standard 6 (1.56 U/ml)	Sample 7	Sample 7
G	Blank	Blank	Sample 8	Sample 8
H	Sample 1	Sample 1	Sample 9	Sample 9

e. Add 100 µl of Sample Diluent in duplicate to the blank wells.

f. Add 100 µl of each prediluted sample in duplicate to the sample wells.

g. Prepare Biotin-Conjugate (see Preparation of Biotin-Conjugate).

h. Add 50 µl of Biotin-Conjugate to all wells.

i. Cover with an adhesive film and incubate at room temperature (18 - 25°C) for 2 hours, if available on a microplate shaker set at 400 rpm.

j. Prepare Streptavidin-HRP (refer to Preparation of Streptavidin-HRP).

k. Remove adhesive film and empty wells. Wash microwell strips 3 times according to point c. of the test

protocol. Proceed immediately to the next step.

l. Add 100 µl of diluted Streptavidin-HRP to all wells, including the blank wells.

m. Cover with an adhesive film and incubate at room temperature (18 - 25°C) for 1 hour, if available on a microplate shaker set at 400 rpm.

n. Remove adhesive film and empty wells. Wash microwell strips 3 times according to point c. of the test protocol. Proceed immediately to the next step.

o. Pipette 100 µl of TMB Substrate Solution to all wells.

p. 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 protocol) 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.

q. 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.

r. 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.

Note: In case of incubation without shaking the obtained O.D. values may be lower than indicated below. Nevertheless the results are still valid.

Test Protocol Summary

1. Predilute serum and plasma samples with Sample Diluent 1:20.
2. Determine the number of microwell strips required.
3. Wash microwell strips twice with Wash Buffer.
4. Standard dilution on the microwell plate: Add 100 µl Sample Diluent, in duplicate, to all standard wells. Pipette 100 µl prepared standard into the first wells and create standard dilutions by transferring 100 µl from well to well. Discard 100 µl from the last wells.

Alternatively external standard dilution in tubes (see 5.1 **External Standard Dilution**): Pipette 100 µl of these standard dilutions in the microwell strips.

5. Add 100 µl Sample Diluent, in duplicate, to the blank wells.
6. Add 100 µl prediluted sample in duplicate, to designated sample wells.
7. Prepare Biotin-Conjugate.
8. Add 50 µl Biotin-Conjugate to all wells.
9. Cover microwell strips and incubate 2 hours at room temperature (18 - 25°C).

10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 3 times with Wash Buffer.
12. Add 100 µl diluted Streptavidin-HRP to all wells.
13. Cover microwell strips and incubate 1 hour at room temperature (18 - 25°C).
14. Empty and wash microwell strips 3 times with Wash Buffer.
15. Add 100 µl of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 10 minutes at room temperature (18 - 25°C).
17. Add 100 µl Stop Solution to all wells.
18. Blank microwell reader and measure colour intensity at 450 nm.

Note: If instructions in this protocol have been followed,serum and plasma samples have been diluted 1:20 and the concentration read from the standard curve must be multiplied by the dilution factor (x 20).

Calculation

− Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20 per cent of the mean value.

− Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the human PSGL-1 concentration on the abscissa. Draw a best fit curve through the points of the graph (a 5-parameter curve fit is recommended).

− To determine the concentration of circulating human PSGL-1 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 PSGL-1 concentration.

− If instructions in this protocol have been followed, serum and plasma samples have been diluted 1:20 and the concentration read from the standard curve must be multiplied by the dilution factor (x 20).

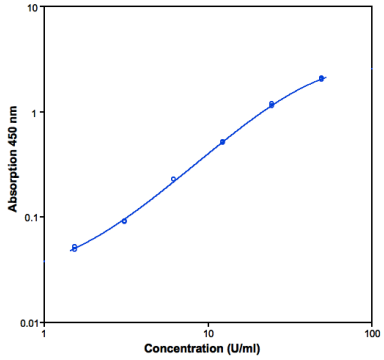
− Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human PSGL-1 levels. Such samples require further external predilution according to expected human PSGL-1 values with Sample Diluent in order to precisely quantitate the actual human PSGL-1 level.

− It is suggested that each testing facility establishes a control sample of known human PSGL-1 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 8. This curve cannot be used to derive test results. Each laboratory must prepare a standard curve for each group of microwell strips assayed.

Representative standard curve for human PSGL-1 ELISA. Human PSGL-1 was diluted in serial 2-fold steps in Sample Diluent. Do not use this standard curve to derive test results. A standard curve must be run for each group of microwell strips assayed.

Figure 8



Standard	Human PSGL-1 Concentration (U/ml)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	50.00	2.041 1.970	2.006	2.5
2	25.00	1.107 1.168	1.138	3.8
3	12.50	0.502 0.506	0.504	0.6
4	6.25	0.223 0.225	0.237	8.1
5	3.13	0.090 0.088	0.089	1.6
6	1.56	0.048 0.051	0.05	4.3
Blank	0.00	0.026 0.022	0.024	11.8

Typical data using the human PSGL-1 ELISA

Measuring wavelength: 450 nm

Reference wavelength: 620 nm

The OD values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects). Furthermore shelf life of the kit may affect enzymatic activity and thus colour intensity. Values measured are still valid.

Evaluation

A panel of 22 serum samples from randomly selected apparently healthy donors (males and females) was tested for human PSGL-1.

The detected human PSGL-1 levels ranged between 329.8 and 762.5 U/ml with a mean level of 451.0 U/ml.

The levels measured may vary with the sample collection used.

Precision

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 PSGL-1. 2 standard curves were run on each plate. Data below show the mean human PSGL-1 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 3.2%.

The mean human PSGL-1 concentration and the coefficient of variation for each sample.

Table 3

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 PSGL-1. 2 standard curves were run on each plate. Data below show the mean human PSGL-1 concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table 4). The calculated overall inter-assay coefficient of variation was 6.6%.

The mean human PSGL-1 concentration and the coefficient of variation of each sample.

Table 4

Sensitivity

The limit of detection of human PSGL-1 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 1.0 U/ml (mean of 6 independent assays).

Specificity

The interference of circulating factors of the immune systeme was evaluated by spiking these proteins at physiologically relevant concentrations into a human PSGL-1 positive serum.

There was no crossreactivity detected.

Recovery

The spike recovery was evaluated by spiking 4 levels of human PSGL-1 into different pooled normal human serum samples. Recoveries were determined in 3 independent experiments with 4 replicates each.

The amount of endogenous human PSGL-1 in unspiked serum was subtracted from the spike values.

The recovery ranged from 83% to 104% with an overall mean recovery of 94%.

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 statements(s) for specific advice
2. Do not mix or substitute reagents with those from other lots or other sources.
3. Do not use kit reagents beyond expiration date on label.
4. Do not expose kit reagents to strong light during storage or incubation.
5. Do not pipette by mouth.
6. Do not eat or smoke in areas where kit reagents or samples are handled.
7. Avoid contact of skin or mucous membranes with kit reagents or specimens.
8. Rubber or disposable latex gloves should be worn while handling kit reagents or specimens.
9. Reagents containing thimerosal as preservative may be toxic if ingested.
10. Avoid contact of substrate solutions 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 reagents.
14. Exposure to acids will inactivate the conjugate.
15. Glass-distilled water or deionized water must be used for reagent preparation.

16. Substrate solutions must be at room temperature prior to use.
17. Decontaminate and dispose specimens and all potentially contaminated materials as if 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 cross-contamination 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. Completely empty wells before dispensing fresh Wash Buffer, 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 immunotherapy has significantly increased the number of patients with human anti-mouse IgG antibody (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 Diluent.