Version 20-04/24

User's Manual

Human TIE-2 ELISA

REF **DEIA4811**

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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.

Creative Diagnostics

Address: 45-1 Ramsey Road, Shirley, NY 11967, USA

Tel: 1-631-624-4882 (USA) 44-161-818-6441 (Europe) **Fax: 1-631-938-8221**

Email: info@creative-diagnostics.com **Web: www.creative-diagnostics.com**

PRODUCT INFORMATION

Intended Use

The human TIE-2 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of human TIE-2. **The human TIE-2 ELISA is for research use only. Not for diagnostic or therapeutic procedures.**

General Description

The tyrosine kinase receptor TIE-2 was initially identified as a specific endothelial growth factor receptor that mediated several properties of endothelial cells under both physiologcal and pathological conditions. Angiopoietins are the natural ligands of TIE-2 and they induce TIE-2-depending signalling, including survival and apoptosis of endothelial cells, vascular permeability and regulate capillary sprouting. In general, the outcome of tie receptor signalling depends on which vascular bed is involved, and crosstalk between different VEGFs has an important modulating effect on the properties of the ligands. TIE-2 plays an important role in several vascular diseases, known as vascular malformation (e.g. venous malformation). Independent of angiogenesis and its involvement in lymphangiogenesis, TIE-2 maintains a long-term, quiescent population of hematopoietc stems cells in the bone marrow. In a tumor model, a subset of monocytes was found to be positve for TIE-2 and that they have an important function in paracrine support of nascent blood vessels.

During cancer formation and spreading, TIE-2 was found to be overexpressed in tumor vessels. However, outsite the vascular compartment TIE-2 is expressed in several types of cancer including leukemia and in gastric tumors, breast tumors and gliomas. In gliomas, TIE-2 expression in the neoplastic glial cells was significantly associated with progression from a lower to a higher grade where it seems to regulate glioma cell adhesion to extracellular matrix.

Together with the reported fact that malignant gliomas epress high levels of Ang1, suggest the existence of an autocrine loop for cell matrix interaction. The possible role of TIE-2 in tumors is more currently under investigation. Because it is expressed in several cellular lineages insite the tumor, the receptor may be an attractive target for cancer therapy.

A natural occuring soluble TIE-2 receptor fragment of 75 kDa (sTIE-2) is generated by shedding. Soluble TIE-2 inhibits angiopoietin-mediated TIE-2 phosphorylation and antiapoptosis. TIE-2 shedding is mediated by PI2K/Akt and p38 MAPK. The serum levels of sTIE-2 in patients is in the lower ng/ml range and dependent of the disease. Soluble TIE-2 was also measured in preterm infants including the ROP syndrome (retinopathy of prematurity).

Elevated plasma levels in active ROP patients were observed for sTIE-2. In experimental tumor mouse models sTIE-2 can be used for tumor regression and prolonged the tumor-free survival in 80% of the animals.

Principles of Testing

1. An anti-human TIE-2 coating antibody is adsorbed onto microwells.
2. Human TIE-2 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-human TIE-2 antibody is added and binds to human TIE-2 captured by the first antibody.
3. Following incubation unbound biotin-conjugated anti-human TIE-2 antibody is removed during a wash step. StreptavidinHRP is added and binds to the biotin-conjugated antihuman TIE-2 antibody.
4. Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution

reactive with HRP is added to the wells.

5. A colored product is formed in proportion to the amount of human TIE-2 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 7 human TIE-2 standard dilutions and human TIE-2 sample concentration determined.

Reagents And Materials Provided

1. 1 aluminium pouch with a **Antibody Coated Microtiter Strips** with polyclonal antibody to human TIE-2
2. 1 vial (70 µl) **Biotin-Conjugate** anti-human TIE-2 polyclonal antibody
3. 1 vial (150 µl) **Streptavidin-HRP**
4. 2 vials human TIE-2 **Standard** lyophilized, 20 ng/ml upon reconstitution
5. 1 vial (12 ml) **Sample Diluent**
6. 1 vial (5 ml) **Assay Buffer Concentrate** 20× (PBS with 1% Tween 20 and 10% BSA)
7. 1 bottle (50 ml) **Wash Buffer Concentrate** 20× (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 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 (EDTA, citrate, 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 samples.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive human TIE-2. 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 3 times, and the human TIE-2 levels determined. There was no significant loss of human TIE-2 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 TIE-2 level determined after 24 h. There was no significant loss of human TIE-2 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. The 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. The Assay Buffer (1×) 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. **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-Conjugate** solution with Assay Buffer (1×) in a clean plastic tube as needed according to the following table:

Number of Strips	Biotin-Conjugate (ml)	Assay Buffer 1× (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 1:100 dilution of the concentrated Streptavidin-HRP solution with Assay Buffer (1×) in a clean plastic tube as needed according to the following table:

Number of Strips	Streptavidin- HRP (ml)	Assay Buffer 1× (ml)
1-6	0.06	5.94
1-12	0.12	11.88

5. **Human TIE-2 Standard**

Reconstitute **human TIE-2 standard** by addition of distilled water.

Reconstitution volume is stated in the Quality Control Sheet. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 20 ng/ml). Allow the standard to reconstitute for 10-30 minutes. Mix well prior to making dilutions.

After usage remaining standard cannot be stored and has to be discarded. Standard dilutions can be prepared directly on the microwell plate (see **Assay Procedure 3**) or alternatively in tubes.

6. **External Standard Dilution**

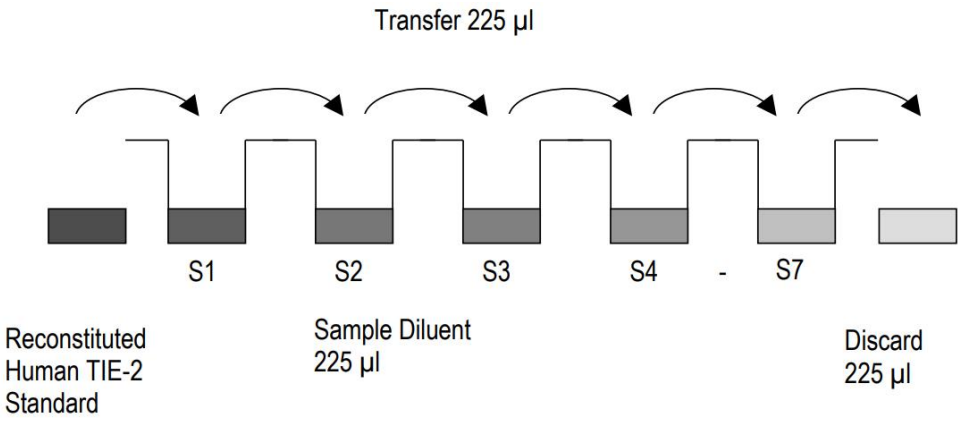
Label 7 tubes, one for each standard point:

S1, S2, S3, S4, S5, S6, S7

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 of standard = 20 ng/ml) into the first tube, labelled S1, and mix (concentration of standard 1 = 10 ng/ml).

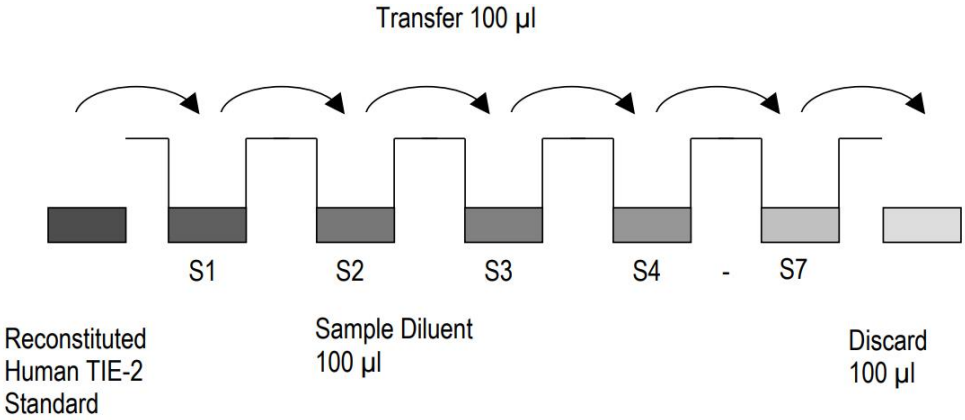
Pipette 225 µl of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure). Sample Diluent serves as blank.



Assay Procedure

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

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 5**, concentration = 20.00 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 = 10.00 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 5 times, creating two rows of human TIE-2 standard dilutions ranging from 10.00 to 0.16 ng/ml. Discard 100 µl of the contents from the last microwells (G1, G2) used.



In case of an external standard dilution, pipette 100 µl of these standard dilutions (S1-S7) in the standard wells according to Table.

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

	1	2	3	4
A	Standard 1 (10.00 ng/ml)	Standard 1 (10.00 ng/ml)	Sample 1	Sample 1
B	Standard 2 (5.00 ng/ml)	Standard 2 (5.00 ng/ml)	Sample 2	Sample 2
C	Standard 3 (2.50 ng/ml)	Standard 3 (2.50 ng/ml)	Sample 3	Sample 3
D	Standard 4 (1.25 ng/ml)	Standard 4 (1.25 ng/ml)	Sample 4	Sample 4
E	Standard 5 (0.63 ng/ml)	Standard 5 (0.63 ng/ml)	Sample 5	Sample 5
F	Standard 6 (0.31 ng/ml)	Standard 6 (0.31 ng/ml)	Sample 6	Sample 6
G	Standard 7 (0.16ng/ml)	Standard 7 (0.16 ng/ml)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

4. Add 100 µl of **Sample Diluent** in duplicate to the **blank wells**.
5. Add 90 µl of **Sample Diluent** to the **sample wells**.
6. Add 10 µl of each **sample** in duplicate to the **sample wells**.
7. Prepare **Biotin-Conjugate** (see to **Reagents Preparation 3**).
8. Add 50 µl of **Biotin-Conjugate** to all wells.
9. Cover with an adhesive film and incubate over night at 4°C, if available on a microplate shaker.
10. Prepare **Streptavidin-HRP** (refer to **Reagents Preparation 4**).
11. Remove adhesive film and empty wells. **Wash** microwell strips 6 times according to point 2. of the test protocol. Proceed immediately to the next step.
12. Add 100 µl of diluted **Streptavidin-HRP** to all wells, including the **blank wells**.
13. Cover with an adhesive film and incubate at room temperature (18-25°C) for 1 hour, if available on a microplate shaker.
14. Remove adhesive film and empty wells. Wash microwell strips 6 times according to point b. of the test protocol. Proceed immediately to the next step.
15. Pipette 100 µl of **TMB Substrate Solution** to all wells.
16. Incubate the microwell strips at room temperature (18-25°C) for about 10 min. Avoid direct exposure to intense light. **The color development on the plate should be monitored and the substrate reaction stopped (see next step) before positive wells are no longer properly recordable. Determination of the ideal time period for color 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 color. Alternatively the color 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.
17. 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

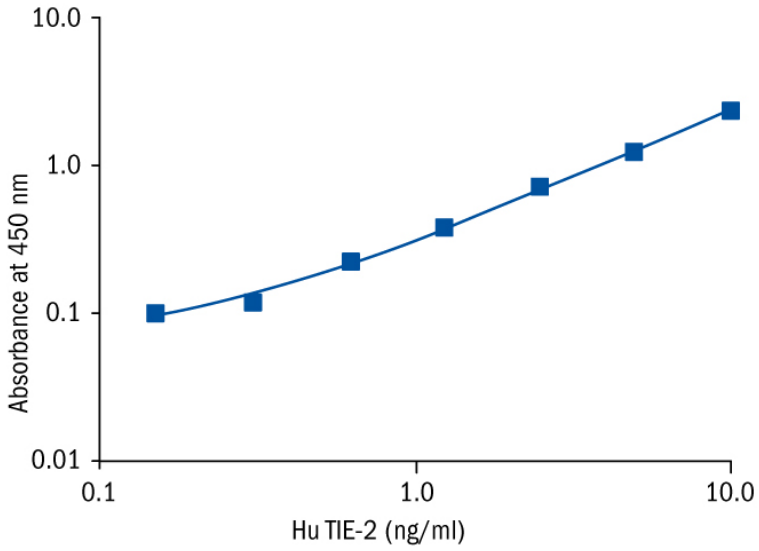
microwell strips are stored at 2-8°C in the dark.

18. 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 TIE-2 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 TIE-2 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 TIE-2 concentration.
4. If instructions in this protocol have been followed samples have been diluted 1:10 (10 µl sample + 90 µl Sample Diluent), the concentration read from the standard curve must be multiplied by the dilution factor (×10).
5. Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low human TIE-2 levels. Such samples require further external predilution according to expected human TIE-2 values with Sample Diluent in order to precisely quantitate the actual human TIE-2 level.
6. It is suggested that each testing facility establishes a control sample of known human TIE2 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 EDTA, citrate and heparin plasma samples from randomly selected apparently healthy donors (males and females) were tested for human TIE-2. The levels measured may vary with the sample collection used. For detected human TIE-2 levels see Table.

Sample Matrix	Number of Samples Evaluated	Range (ng/ml)	Mean (ng/ml)	Standard Deviation (ng/ml)
Serum	40	2.9 – 78.4	33.6	16.9
Plasma (EDTA)	40	5.0 – 54.4	24.1	10.0
Plasma (Citrate)	40	2.1 – 45.1	26.4	10.8
Plasma (Heparin)	40	9.1 – 47.5	27.2	9.3

Detection Range

0.16-10.0 ng/ml

Detection Limit

0.03 ng/ml

Sensitivity

The limit of detection of human TIE-2 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 0.03 ng/ml (mean of 6 independent assays).

Specificity

The assay detects both natural and recombinant human TIE-2.

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into serum.

There was no cross-reactivity detected, notably not with TIE-1.

Linearity

Serum samples with different levels of human TIE-2 were analyzed at serial 2 fold dilutions with 4 replicates each. The recovery ranged from 96.8% to 133.7% with an overall recovery of 108.3% (see Table).

Sample	Dilution	Expected Human TIE-2 Concentration (ng/ml)	Observed Human TIE-2 Concentration (ng/ml)	Recovery of Expected Human TIE-2 Concentration (%)
1	1:2		45.5	
	1:4	22.7	22.0	96.8
	1:8	11.0	11.5	105.0
	1:16	5.8	6.3	108.6
2	1:2		43.3	
	1:4	21.6	21.4	99.0
	1:8	10.7	10.6	98.7
	1:16	5.3	5.3	100.5
3	1:2		24.2	
	1:4	12.1	15.0	124.4
	1:8	7.5	8.4	111.9
	1:16	4.2	4.6	108.8
4	1:2		27.5	
	1:4	13.8	18.4	133.7
	1:8	9.2	10.4	112.9
	1:16	5.2	5.2	99.7

Recovery

The spiking recovery was evaluated by spiking 4 levels of human TIE-2 into serum. Recoveries were determined in 3 independent experiments with 4 replicates each. The amount of endogenous human TIE-2 in unspiked serum was subtracted from the spike values. The recovery ranged from 73% to 102% with an overall mean recovery of 87%.

Reproducibility

1. **Intra-assay**

Reproducibility within the assay was evaluated in 3 independent experiments. Each assay was carried out with 6 replicates of 6 serum samples containing different concentrations of human TIE-2. 2 standard curves were run on each plate. Data below show the mean human TIE-2 concentration and the coefficient of variation for each sample (see Table). The calculated overall intra-assay coefficient of variation was 7.1%.

The mean human TIE-2 concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Human TIE-2 Concentration (ng/ml)	Coefficient of Variation (%)
1	1	1.8	10.8
	2	2.1	10.9
	3	2.0	9.7
2	1	21.6	1.6
	2	21.5	6.0
	3	23.8	1.9
3	1	11.1	7.3
	2	11.7	6.3
	3	12.8	3.9
4	1	2.1	9.9
	2	2.3	9.3
	3	2.5	5.7
5	1	6.1	8.4
	2	5.7	5.7
	3	6.0	5.6
6	1	29.9	10.9
	2	27.3	5.8
	3	27.9	8.3

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 6 serum samples containing different concentrations of human TIE-2. 2 standard curves were run on each plate. Data below show the mean human TIE-2 concentration and the coefficient of variation calculated on 18 determinations of each sample (see Table). The calculated overall inter-assay coefficient of variation was 6.3%.

The mean human TIE-2 concentration and the coefficient of variation of each sample

Sample	Mean Human TIE-2 Concentration (ng/ml)	Coefficient of Variation (%)
1	2.0	8.3
2	22.3	5.9
3	11.9	6.9
4	2.3	8.7
5	5.9	3.4
6	28.4	4.9

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 samples.
9. Rubber or disposable latex gloves should be worn while handling kit reagents or samples.
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.