



Mouse IL-6 ELISA

For quantitative detection of mouse IL-6 in cell culture supernatant, serum, EDTA and citrate plasma samples.

For Research Use Only. Not For Use In Diagnostic Procedures.

Catalog Number: 61-IL6MS-E01

Size: 96 Tests

Version: A.0 (30) - ALPCO 2.0

1 Intended Use

The Mouse IL-6 ELISA is an enzyme-linked immunosorbent assay for the quantitative detection of mouse IL-6. The mouse IL-6 ELISA is for Research Use Only. Not for diagnostic or therapeutic procedures.

2 Summary

Interleukin-6 (IL-6) is a multi-functional cytokine that regulates immune responses, acute phase reactions and hematopoiesis and may play a central role in host defense mechanisms. IL-6 is usually not produced constitutively by normal cells, but its expression is readily induced by a variety of cytokines, lipopolysaccharide or viral infections. IL-6 is a pleiotropic cytokine produced by a variety of cells. It acts on a wide range of tissues, exerting growth-induction, growth-inhibition, and differentiation respectively, depending on the nature of the target cells.

Research has demonstrated that IL-6 is involved in:

- the induction of B-cell differentiation
- the induction of acute phase proteins in liver cells
- growth promotion of myeloma/plasmacytoma/hybridoma cells
- induction of IL-2 and IL-2 receptor expression
- proliferation and differentiation of T cells
- inhibition of cell growth of certain myeloid leukemic cell lines and induction of their differentiation to macrophages
- enhancement of IL-3-induced multipotential colony cell formation in hematopoietic stem cells and induction of maturation of megakaryocytes as a thrombopoietic factor
- induction of mesangial cell growth
- induction of neural differentiation of PC 12 cells
- induction of keratinocyte growth

3 Principles of the Test

An anti-mouse IL-6 coating antibody is adsorbed onto microwells.

Figure 1

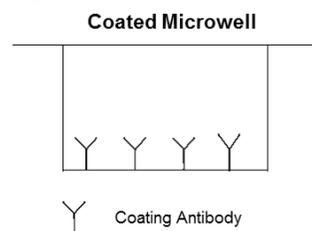
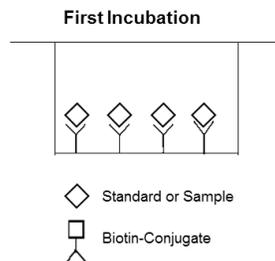
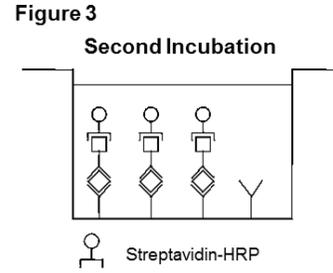


Figure 2

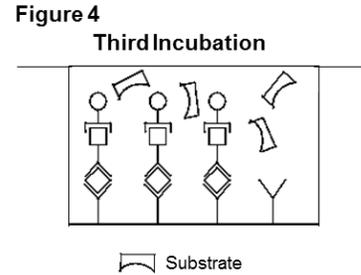


Mouse IL-6 present in the sample or standard binds to antibodies adsorbed to the microwells. A biotin-conjugated anti-mouse IL-6 antibody is added and binds to mouse IL-6 captured by the first antibody.

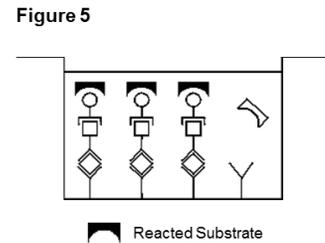
Following incubation unbound biotin-conjugated anti-mouse IL-6 antibody is removed during a wash step. Streptavidin-HRP is added and binds to the biotin-conjugated anti-mouse IL-6 antibody.



Following incubation unbound Streptavidin-HRP is removed during a wash step, and substrate solution reactive with HRP is added to the wells.



A colored product is formed in proportion to the amount of mouse IL-6 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 mouse IL-6 standard dilutions and the mouse IL-6 sample concentration determined.



4 Reagents Provided

4.1 Reagents for mouse IL-6 ELISA (96 tests)

- 1 aluminum pouch with a Microwell Plate coated with monoclonal antibody to mouse IL-6
- 1 vial (60 µL) Biotin-Conjugate anti-mouse IL-6 monoclonal antibody
- 1 vial (150 µL) Streptavidin-HRP
- 2 vials mouse IL-6 Standard lyophilized, 4 ng/mL upon reconstitution
- 1 vial (12 mL) Sample Diluent
- 1 vial (18 mL) Assay Buffer
- 1 bottle (50 mL) Wash Buffer Concentrate 20x (PBS with 1% Tween 20)
- 1 vial (15 mL) Substrate Solution (Tetramethylbenzidine)
- 1 vial (15 mL) Stop Solution (1M Phosphoric acid)
- 4 Adhesive Films

5 Storage Instructions – ELISA Kit

Store kit reagents between 2°C and 8°C. Immediately after use remaining reagents should be returned to cold storage (2° to 8°C). Expiry of the kit and reagents is stated on labels.

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, this reagent is not contaminated by the first handling.

6 Sample Collection and Storage Instructions

Cell culture supernatant, serum and plasma (EDTA, citrate) were tested with this assay. Other body fluids 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 samples.

Samples should be aliquoted and must be stored frozen at -20°C to avoid loss of bioactive mouse IL-6. If samples are to be run within 24 hours, they may be stored at 2° to 8°C (for sample stability refer to section 13.5).

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

7 Materials Required But Not Provided

- 5 mL and 10 mL graduated pipettes
- 5 µL to 1000 µL adjustable single channel micropipettes with disposable tips
- 50 µL to 300 µL 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 wavelength)
- Microplate shaker capable of 400 rpm
- Distilled or deionized water
- Statistical calculator with program to perform regression analysis
- Timer

8 Precautions for Use

- All chemicals should be considered as potentially hazardous. It is therefore recommended 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 coat, 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 safety data sheet(s) and/or safety statement(s) for specific advice.
- Reagents are intended for research use only and are not for use in diagnostic or 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 samples.
- Disposable gloves should be worn while handling kit reagents or samples.

- 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 samples, which may invalidate the test, use disposable pipette tips and/or pipettes.
- Use clean, dedicated reagent trays for dispensing the conjugate and substrate reagent.
- Exposure to acid inactivates the conjugate.
- Distilled water or deionized water must be used for reagent preparation.
- Substrate solution must be at room temperature prior to use.
- Decontaminate and dispose of 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.
- Liquid waste 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

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

9.1 Wash Buffer (1x)

1. Pour entire contents (50 mL) of the Wash Buffer Concentrate (20x) into a clean 1000 mL graduated cylinder. Bring to a final volume of 1000 mL with distilled or deionized water.
2. Mix gently to avoid foaming.
3. Transfer to a clean wash bottle and store at 2°C to 25°C. Please note that Wash Buffer (1x) is stable for 30 days.
4. 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

9.2 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 (1x) in a clean plastic tube as needed according to the following table:

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

9.3 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 (1x) in a clean plastic tube as needed according to the following table:

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

9.4 Mouse IL-6 Standard

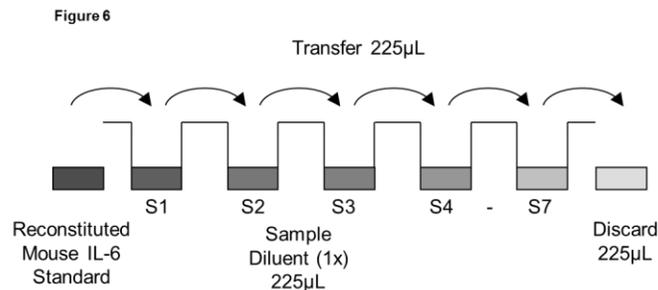
Reconstitute **mouse IL-6 standard** by addition of distilled water. Reconstitution volume is stated on the label of the standard vial. Swirl or mix gently to insure complete and homogeneous solubilization (concentration of reconstituted standard = 4 ng/mL). Allow the reconstituted standard to sit for 10-30 minutes. Mix well prior to making dilutions.

The standard has to be used immediately after reconstitution and cannot be stored.

Standard dilutions can be prepared directly on the microwell plate (see section 10.3) or alternatively in tubes (see section 9.4.1).

9.4.1 External Standard Dilution

1. Label 7 tubes, one for each standard: S1, S2, S3, S4, S5, S6, S7
2. Then prepare 1:2 serial dilutions for the standard curve as follows:
Pipette 225 μ L of Sample Diluent into each tube.
3. Pipette 225 μ L of reconstituted standard (concentration = 4000 pg/mL) into the first tube, labelled S1, and mix (concentration of standard 1 = 2000 pg/mL).
4. Pipette 225 μ L of this dilution into the second tube, labelled S2, and mix thoroughly before the next transfer.
5. Repeat serial dilutions 5 more times thus creating the points of the standard curve (see Figure 6).
6. Sample Diluent serves as blank.



10 Test Protocol

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.

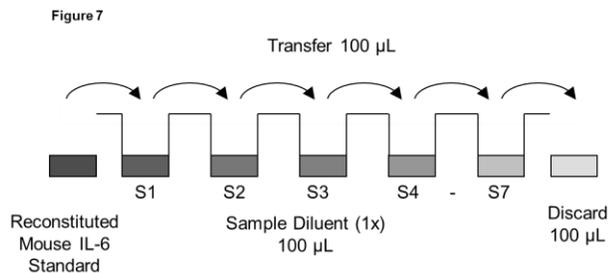
- Wash the microwell strips twice with approximately 400 μL **Wash Buffer** per well with thorough aspiration of microwell contents between washes. Allow the Wash Buffer to sit in the wells for about **10 – 15 seconds** before aspiration. Take care not to scratch the surface of the microwells.

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 - see section 9.4.1):

- Add 100 μL of Sample Diluent in duplicate to all **standard wells**.
- Pipette 100 μL of prepared **standard** (see Preparation of Standard, section 9.4), concentration = 4000 pg/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 = 2000 pg/mL), and transfer 100 μL to wells B1 and B2, respectively (see Figure 7).
- Take care not to scratch the inner surface of the microwells.
- Continue this procedure 5 times, creating two rows of mouse IL-6 standard dilutions, ranging from 2000 to 31.3 pg/mL. Discard 100 μL of the contents from the last microwells (G1, G2) used.



- In case of an **external standard dilution** (see section 9.4.1), pipette 100 μL of these standard dilutions (S1 - S7) in the standard wells according to Table 1.

Table 1

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

	1	2	3	4
A	Standard 1 (2000.0 pg/mL)	Standard 1 (2000.0 pg/mL)	Sample 1	Sample 1
B	Standard 2 (1000.0 pg/mL)	Standard 2 (1000.0 pg/mL)	Sample 2	Sample 2
C	Standard 3 (500.0 pg/mL)	Standard 3 (500.0 pg/mL)	Sample 3	Sample 3
D	Standard 4 (250.0 pg/mL)	Standard 4 (250.0 pg/mL)	Sample 4	Sample 4
E	Standard 5 (125.0 pg/mL)	Standard 5 (125.0 pg/mL)	Sample 5	Sample 5
F	Standard 6 (62.5pg/mL)	Standard 6 (62.5 pg/mL)	Sample 6	Sample 6
G	Standard 7 (31.3 pg/mL)	Standard 7 (31.3 pg/mL)	Sample 7	Sample 7
H	Blank	Blank	Sample 8	Sample 8

4. Add 100 μ L of Sample Diluent in duplicate to the blank wells.
5. Add 50 μ L of **Sample Diluent** to the **sample wells**.
6. Add 50 μ L of each **sample** in duplicate to the **sample wells**.
7. Prepare **Biotin-Conjugate** (see Preparation of Biotin-Conjugate, section 9.2).
8. Add 50 μ L of **Biotin-Conjugate** to all wells.
9. Cover with an adhesive film and incubate at room temperature (18 to 25°C) for 2 hours on a microplate shaker set at 400 rpm. (**Shaking is absolutely necessary for optimal test performance.**)
10. Prepare **Streptavidin-HRP** (refer to Preparation of Streptavidin-HRP, section 9.3).
11. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to section 10.2 of the protocol. Proceed immediately to the next step.
12. Add 100 μ L of diluted **Streptavidin-HRP** to all wells, including the blank wells.
13. Cover with an adhesive film and incubate at room temperature (18° to 25°C) for 1 hour on a microplate shaker set at 400 rpm. (**Shaking is absolutely necessary for an optimal test performance.**)
14. Remove adhesive film and empty wells. **Wash** microwell strips 5 times according to section 10.2 of the protocol. Proceed immediately to the next step.
15. Pipette 100 μ L of **TMB Substrate Solution** to all wells.
16. Incubate the microwell strips at room temperature (18° to 25°C) for about 30 min. Avoid direct exposure to intense light.

The color 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 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 μ L 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 spectrophotometer using 450 nm as the primary wavelength (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.

11 Calculation of Results

- Calculate the average absorbance values for each set of duplicate standards and samples. Duplicates should be within 20% of the mean value.
- Create a standard curve by plotting the mean absorbance for each standard concentration on the ordinate against the mouse IL-6 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 mouse IL-6 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 mouse IL-6 concentration.
- **If instructions in this protocol have been followed, samples have been diluted 1:2 (50 μ L sample + 50 μ L Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x2).**
- **Calculation of samples with a concentration exceeding standard 1 may result in incorrect, low mouse IL-6 levels. Such samples require further external predilution, according to expected mouse IL-6 values, with Sample Diluent in order to precisely quantitate the actual mouse IL-6 level.**
- It is suggested that each testing facility establishes a control sample of known mouse IL-6 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.

Figure 8

Representative standard curve for mouse IL-6 ELISA. Do not use for calculation of results. Mouse IL-6 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.

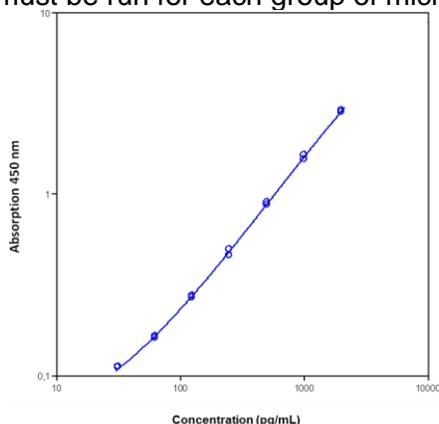


Table 2

Typical data using the mouse IL-6 ELISA
 Measuring wavelength: 450 nm
 Reference wavelength: 620 nm

Standard	Mouse IL-6 Concentration (pg/mL)	O.D. at 450 nm	Mean O.D. at 450 nm	C.V. (%)
1	2000	2.821 2.908	2.864	1.5
2	1000	1.645 1.555	1.600	2.8
3	500	0.904 0.878	0.891	1.5
4	250	0.461 0.498	0.480	3.9
5	125	0.275 0.271	0.273	0.7
6	62.5	0.162 0.166	0.164	1.3
7	31.3	0.111 0.113	0.112	0.6
Blank	0	0.052 0.054	0.053	1.8

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 color intensity. Values measured are still valid.

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 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.

13 Performance Characteristics

13.1 Analytical Sensitivity

The limit of detection of mouse IL-6 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 6.5 pg/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 8 serum samples containing different concentrations of mouse IL-6. Two standard curves were run on each plate. Data below show the mean mouse IL-6 concentration and the coefficient of variation for each sample (see Table 3). The calculated overall intra-assay coefficient of variation was 5.0%.

Table 3

The mean mouse IL-6 concentration and the coefficient of variation for each sample

Sample	Experiment	Mean Mouse IL-6 Concentration (pg/mL)	Coefficient of Variation (%)
1	1	2942	3.2
	2	3314	3.7
	3	2811	4.4
2	1	568	4.3
	2	673	5.1
	3	570	9
3	1	174	5.4
	2	195	2.2
	3	157	3.8
4	1	62	10.5
	2	79	7.1

	3	67	4.1
5	1	1020	4.1
	2	1089	2.2
	3	1075	3.2
6	1	542	6.3
	2	614	4.1
	3	520	6.6
7	1	274	3
	2	305	5.5
	3	263	3.9
8	1	142	8.7
	2	162	4.9
	3	134	4

13.2.2 Inter-assay

Assay to assay reproducibility within one laboratory was evaluated. Each assay was carried out with 6 replicates of 8 serum samples containing different concentrations of mouse IL-6. Two standard curves were run on each plate. Data below show the mean mouse IL-6 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 8.9%.

Table 4

The mean mouse IL-6 concentration and the coefficient of variation of each sample:

Sample	Mean Mouse IL-6 Concentration (pg/mL)	Coefficient of Variation (%)
1	3022	8.6
2	604	9.9
3	175	10.6
4	69	12.0
5	1061	3.5
6	558	8.8
7	280	7.9
8	146	10.0

13.3 Spike Recovery

The spike recovery was evaluated by spiking 3 levels of mouse IL-6 into serum, plasma and cell culture supernatant. Recoveries were determined with 4 replicates each. The unspiked serum, plasma, cell culture supernatant was used as blank in these experiments. For recovery data see Table 5.

Table 5

Sample matrix	Spike high (%)	Spike medium (%)	Spike low (%)
Serum	89% (87-91)	74% (72-76)	96% (79-112)
Plasma (EDTA)	85% (71-100)	91% (88-97)	123% (112-134)
Plasma (citrate)	63% (62-65)	60% (59-61)	101% (99-102)
Cell culture supernatant	93% (81-105)	111% (110-113)	103% (96-110)

13.4 Dilution Parallelism

Serum, plasma (EDTA, citrate) and cell culture supernatant samples with different levels of mouse IL-6 were analyzed at serial 2-fold dilutions with 4 replicates each. For recovery data see Table 6.

Table 6

Sample Matrix	Recovery	
	Mean (%)	Range (%)
Serum	100%	91 - 114
Plasma (EDTA)	106%	99 - 117
Plasma (citrate)	135%	124 - 149
Cell culture supernatant	95%	90 - 98

13.5 Sample Stability

13.5.1 Freeze-Thaw Stability

Aliquots of spiked serum samples were stored at -20°C and thawed 5 times, and the mouse IL-6 levels determined. There was no significant loss of mouse IL-6 immunoreactivity detected by freezing and thawing.

13.5.2 Storage Stability

Aliquots of spiked serum samples were stored at -20°C, 2-8°C, room temperature (RT) and at 37°C, and the mouse IL-6 level determined after 24 hours. There was no significant loss of mouse IL-6 immunoreactivity detected during storage at -20°C, 2-8°C or RT.

A significant loss of mouse IL-6 immunoreactivity was detected during storage at 37°C after 24 hours.

13.6 Analytical Specificity

The interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a mouse IL-6 positive serum.

There was no cross-reactivity observed with mouse IL-17, IL-2, IFN-gamma, TNF-alpha, IL-5, IL-1-alpha, GM-CSF, IL-4, or IL-10.

13.7 Calibration

The immunoassay is calibrated with highly purified recombinant mouse IL-6 which has been evaluated against the International Reference Standard NIBSC 93/730 and has been shown to be equivalent. NIBSC 93/730 is quantitated in International Units (IU), where 1IU corresponds to 10 pg mouse IL-6.

14 Reagent Preparation Summary

14.1 Wash Buffer (1x)

Add Wash Buffer Concentrate 20x (50 mL) to 950 mL distilled water.

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

14.2 Biotin-Conjugate

Make a 1:100 dilution of Biotin-Conjugate in Assay Buffer (1x):

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

14.3 Streptavidin-HRP

Make a 1:100 dilution of Streptavidin-HRP in Assay Buffer (1x):

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

14.4 Mouse IL-6 Standard

Reconstitute lyophilized mouse IL-6 standard with distilled water. (Reconstitution volume is stated on the label of the standard vial.)

15 Test Protocol Summary

1. Determine the number of microwell strips required.
2. Wash microwell strips twice with Wash Buffer.
3. Standard dilution on the microwell plate:
 - a. 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.
 - b. Alternatively, external standard dilution in tubes (see section 9.4.1): pipette 100 µL of these standard dilutions into the microwells strips.
4. Add 100 µL Sample Diluent, in duplicate, to the blank wells.
5. Add 50 µL Sample Diluent to sample wells.
6. Add 50 µL 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° to 25°C) on a microplate shaker set at 400 rpm. (Shaking is absolutely necessary for an optimal test performance.)
10. Prepare Streptavidin-HRP.
11. Empty and wash microwell strips 5 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° to 25°C) on a microplate shaker set at 400 rpm. (Shaking is absolutely necessary for an optimal test performance.)
14. Empty and wash microwell strips 5 times with Wash Buffer.
15. Add 100 µL of TMB Substrate Solution to all wells.
16. Incubate the microwell strips for about 30 minutes at room temperature (18°to 25°C).
17. Add 100 µL Stop Solution to all wells.
18. Blank microwell reader and measure color intensity at 450 nm.

Note: If instructions in this protocol have been followed, samples have been diluted 1:2 (50 µL sample + 50 µL Sample Diluent) and the concentration read from the standard curve must be multiplied by the dilution factor (x2).