INSTRUCTIONS

Rat IL-6 ELISA Kit

ER3IL6 ER3IL62 ER3IL65

1368.5

Number	Description
ER3IL6	**Rat Interleukin-6 (IL-6) ELISA Kit,** sufficient reagents for 96 determinations
ER3IL62	**Rat Interleukin-6 (IL-6) ELISA Kit,** sufficient reagents for 2 × 96 determinations
ER3IL65	**Rat Interleukin-6 ELISA Kit,** sufficient reagents for 5 × 96 determinations

Kit Contents	ER3IL6	ER3IL62	ER3IL65
Anti-Rat IL-6 Pre-coated 96-well Strip Plate	1 each	2 each	5 each
Lyophilized Recombinant Rat IL-6 Standard	2 vials	4 vials	5 vials
Standard Diluent	12mL	2 × 12mL	55mL
Biotinylated Antibody Reagent	12mL	2 × 12mL	55mL
20X Wash Buffer	55mL	2 × 55mL	250mL
Streptavidin-HRP Concentrate	75µL	2 × 75µL	250µL
Streptavidin-HRP Dilution Buffer	13mL	2 × 13mL	70mL
TMB Substrate	13mL	2 × 13mL	5 × 13mL
Stop Solution, contains 2N sulfuric acid	6mL	2 × 6mL	30mL
Adhesive Plate Covers	6 each	12 each	30 each

For research use only – not for use in diagnostic procedures.

Storage: For maximum stability, store in a non-defrosting -20ºC freezer and refer to the expiration date for frozen storage on the label. Kit is shipped with dry ice.

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Introduction

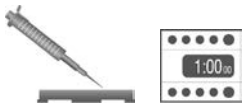
The Thermo Scientific Rat Interleukin-6 (IL-6) ELISA is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of rat IL-6 in culture supernatants, EDTA plasma, sodium citrate plasma, heparin plasma and serum.

Procedure Summary

1. Add 100µL of standards or samples to each well in duplicate.

2. Cover plate and incubate at room temperature (20-25°C) for 2 hours.

3. Wash plate FIVE times.



4. Add 100µL of Biotinylated Antibody Reagent to each well. Cover plate and incubate at room temperature for 1 hour.

5. Wash plate FIVE times.

6. Add 100µL of prepared Streptavidin-HRP Solution to each well.

7. Cover plate and incubate at room temperature for 30 minutes.

8. Wash plate FIVE times.

9. Add 100µL of TMB Substrate to each well.

10. Develop plate in the dark at room temperature for 30 minutes.

11. Add 50µL of Stop Solution to each well.

12. Measure absorbance on a plate reader at 450nm minus 550nm. Calculate results using graph paper or curve-fitting statistical software.

Additional Materials Required

- Precision pipettors with disposable plastic tips to deliver 5-1000µL
- Plastic pipettes to deliver 5-15mL
- A glass or plastic 2L container to prepare Wash Buffer
- A squirt wash bottle or an automated 96-well plate washer
- 1.5mL polypropylene or polyethylene tubes to prepare standards – do not use polystyrene, polycarbonate or glass tubes
- Disposable reagent reservoirs
- 15mL plastic tube to prepare Streptavidin-HRP Solution
- Microcentrifuge to prepare Streptavidin-HRP Solution
- A standard ELISA reader for measuring absorbance at 450nm and 550nm. If a 550nm filter is not available, the absorbance can be measured at 450nm only. Refer to the instruction manual supplied with the instrument being used.
- Graph paper or a computerized curve-fitting statistical software package

Precautions

- **All samples and reagents must be at room temperature (20-25°C) before use in the assay.**
- Review all instructions carefully and verify all components against the Kit Contents list (page 1) before beginning.
- Do not use a water bath to thaw samples. Thaw samples at room temperature.
- If using a multichannel pipettor, always use a new disposable reagent reservoir.
- Use new disposable pipette tips for each transfer to avoid cross-contamination.
- Use a new adhesive plate cover for each incubation step.

- Once reagents have been added to the plate, take care NOT to let plate DRY at any time during the assay.
- Avoid microbial contamination of reagents.
- Vigorous plate washing is essential.
- Avoid exposing reagents to excessive heat or light during storage and incubation.
- Discard unused kit components. Do not mix reagents from different kit lots.
- Do not use glass pipettes to measure TMB Substrate Solution. Take care not to contaminate the substrate solution. If the solution is blue before use, DO NOT USE IT.
- Individual components may contain antibiotics and preservatives. Wear gloves while performing the assay to avoid contact with samples and reagents. Please follow proper disposal procedures.
- Coated plate wells may appear clear to hazy. Haziness does not impact product performance.

Additional Precautions for the 5-plate Kit

- Dispense only reagent volumes required for the number of plates being used. Do not combine leftover reagents with those reserved for additional plates.
- Use only one bottle of the TMB Substrate Solution per 96-well plate. Do not combine leftover substrate with that reserved for other plates.
- Equilibrate to room temperature only the reagent volumes required for the number of plates being used.
- Use only one vial of standard per 96-well plate.

Sample Preparation

Sample Handling

- Serum; EDTA, heparin and sodium citrate plasma; or culture supernatants may be tested in this ELISA.
- **Dilute samples three-fold in Standard Diluent before assaying.** For example, a three-fold dilution is prepared by adding 50µL of test sample to 100µL of Standard Diluent. Mix thoroughly before assaying.
- If the rat IL-6 concentration possibly exceeds the highest point of the standard curve (i.e., 4000pg/mL), prepare one or more five-fold dilutions of the test sample using the Standard Diluent provided. For example, a five-fold dilution is prepared by adding 50µL of test sample to 200µL of Sample Diluent. Mix thoroughly between dilutions before assaying.
- Use 100µL per well of diluted serum, plasma or culture supernatant.
- Store samples to be assayed within 24 hours at 2-8°C. For long-term storage, aliquot and freeze samples at -70°C. Avoid repeated freeze-thaw cycles when storing samples.
- Test samples and standards must be assayed in duplicate each time the assay is performed.
- Gradually equilibrate samples to room temperature before beginning assay. Do not use a heated water bath to thaw or warm samples. Mix samples by gently inverting tubes.
- If samples are clotted, grossly hemolyzed, lipemic or microbially contaminated, or if there is any question about the integrity of a sample, make a note on the template and interpret results with caution.

Reagent Preparation

For procedural differences when using partial plates, look for **(PP)** throughout these instructions.

Note: When using the 5-plate kit, only one standard per plate is supplied. Therefore, partial plates cannot be used.

Wash Buffer

1. Label a clean glass or plastic two-liter container “Wash Buffer.” The 20X Wash Buffer may have a cloudy appearance.
2. For the 5-plate kit, add 40mL Wash Buffer to 760mL water for each plate used. For the 1- and 2-plate kits, add 50mL of the 20X Wash Buffer bottle to the 2L container and dilute to a final volume of 1L with ultrapure water. Mix thoroughly.

(PP) When using partial plates, store the reconstituted Wash Buffer at 2-8°C.

Note: Wash Buffer must be at room temperature before use in the assay. Do not use Wash Buffer if it becomes visibly contaminated during storage.

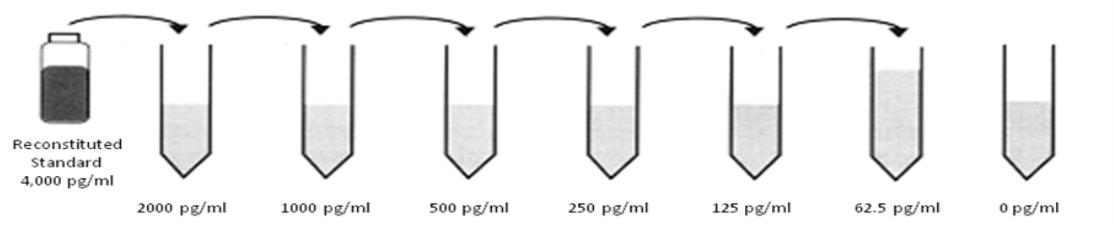
Standards

- **(PP)** Reconstitute and use one vial of the lyophilized standard per partial plate.
- Prepare standards just before use and use within one hour of reconstitution. Do not store reconstituted standards.

1. Reconstitute standard with Standard Diluent. Reconstitution volume is stated on the standard vial label. The standard will dissolve in approximately 1 minute and will be **4000pg/mL, which is the highest point for the standard curve**. Mix by gently inverting vial. Use the Standard Diluent provided to prepare standard curve serial dilutions.

   Note: Prepare the standard curve in Standard Diluent for all sample types; however, if preparing a standard curve without Standard Diluent, perform the curve in a parallel with standard curve reconstituted in standard diluent. If the OD values are within 10% of the mean of both curves, the standards may be prepared without the use of Standard Diluent.
2. Label seven tubes, one for each additional standard curve point: 2000, 1000, 500, 250, 125, 62.5 and 0pg/mL. Prepare 1:2 serial dilutions for the standard curve as follows:
3. Pipette 250µL of Standard Diluent into each tube.
4. Pipette 250µL of the reconstituted standard into the first tube (i.e., 2000pg/mL) and mix.
5. Pipette 250µL of this dilution into the second tube (i.e., 1000pg/mL) and mix.
6. Repeat the serial dilutions (using 250µL) four more times to complete the standard curve points.

Serial Dilutions using 250µL



Assay Procedure

A. Sample Incubation

- **(PP)** Determine the number of strips required. Leave these strips in the plate frame. Tightly seal remaining unused strips in the provided foil pouch with desiccant and store at 2-8°C. After completing assay, retain plate frame for second partial plate. When using the second partial plate, place strips securely in the plate frame.
- Use the Data Template provided to record the locations of the zero standard (blank or negative control), rat IL-6 standards and test samples. Perform seven standard points and one blank in duplicate with each series of unknowns.

1. Add 100µL of reconstituted standards or test samples (diluted three-fold in Standard Diluent, see Sample Preparation – Sample Handling Section) in duplicate to each well. Mix well by gently tapping the plate several times.

   Note: If the rat IL-6 concentration in any sample possibly exceeds the highest point on the standard curve, 4000pg/mL, see Sample Preparation – Sample Dilution section.
2. Carefully cover plate with a new adhesive plate cover. Ensure all edges and strips are sealed tightly by running your thumb over edges and down each strip. Incubate for 2 hours at room temperature, 20-25°C.
3. Carefully remove adhesive plate cover. Wash plate FIVE times with Wash Buffer as described in the Plate Washing Section (Section B).

B. Plate Washing

Note: Automated plate washers may produce sub-optimal results. For best results, perform a manual wash the first time the assay is performed. Automated plate washing may require validation to determine the number of washes necessary to achieve optimal results; typically, 3-5 washes are sufficient.

1. Gently squeeze the long sides of plate frame before washing to ensure all strips securely remain in the frame.
2. Empty plate contents. Use a squirt wash bottle to vigorously fill each well completely with Wash Buffer, then empty plate contents. Repeat procedure two additional times for a total of FIVE washes. Blot plate onto paper towels or other absorbent material.

   For automated washing, aspirate all wells and wash with Wash Buffer, overfilling wells with Wash Buffer. Blot plate onto paper towels or other absorbent material. The number of washes necessary may require optimization.

C. Biotinylated Antibody Reagent Incubation

- If using a multichannel pipettor, **use a new reagent reservoir and pipette tips** when adding the Biotinylated Antibody Reagent. Remove from the vial only the amount required for the number of strips being used. Take care not to touch the samples in wells with the pipette tip when adding the Biotinylated Antibody Reagent.

1. Add 100µL of Biotinylated Antibody Reagent to each well containing sample or standard.
2. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 1 hour at room temperature, 20-25°C.
3. Carefully remove the adhesive plate cover, discard plate contents and wash FIVE times as described in the Plate Washing Section (Section B).

D. Streptavidin-HRP Solution Preparation and Incubation

- Prepare Streptavidin-HRP Solution immediately before use**.** Do not prepare more Streptavidin-HRP than required and do not store prepared Streptavidin-HRP Solution.
- Use a 15mL plastic tube to prepare Streptavidin-HRP Solution.
- If using a multichannel pipettor, use new reagent reservoir and pipette tips when adding the Streptavidin-HRP Solution.

1. Briefly centrifuge the Streptavidin-HRP Concentrate to force entire vial contents to the bottom.
2. **(PP)** Use only the Streptavidin-HRP Solution amount required for the number of strips being used. For each strip, mix 2.5µL of Streptavidin-HRP Concentrate with 1mL of Streptavidin-HRP Dilution Buffer. Store Streptavidin-HRP Concentrate reserved for additional strips at 2-8°C.

   For one complete 96-well plate, add 30µL of Streptavidin-HRP Concentrate to 12mL of Streptavidin-HRP Dilution Buffer and mix gently.
3. Add 100µL of prepared Streptavidin-HRP Solution to each well.
4. Carefully attach a new adhesive plate cover, ensuring all edges and strips are tightly sealed. Incubate plate for 30 minutes at room temperature, 20-25°C.
5. Carefully remove the adhesive plate cover, discard plate contents and wash FIVE times as described in the Plate Washing Section (Section B).

E. Substrate Incubation and Stop Step

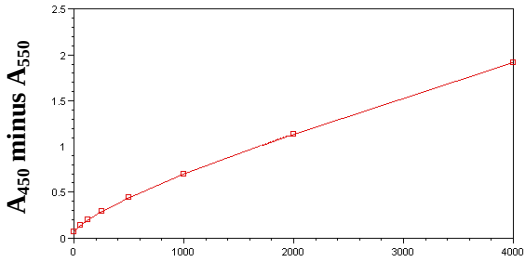
- Use new disposable reagent reservoirs when adding TMB Substrate and Stop Solution.
- Dispense from bottle ONLY amount required, 100µL per well, for the number of wells being used. Do not use a glass pipette to measure the TMB Substrate.
- **(PP)** Do not combine leftover substrate with that reserved for the second partial plate. Take care not to contaminate remaining TMB Substrate.

1. Pipette 100µL of TMB Substrate into each well.

2. Allow the color reaction to develop at room temperature in the dark for 30 minutes. The substrate reaction yields a blue solution that turns yellow when Stop Solution is added.
3. After 30 minutes, stop the reaction by adding 50µL of Stop Solution to each well.

F. Absorbance Measurement

- Evaluate the plate within 15 minutes of stopping the reaction. Measure the absorbance on an ELISA plate reader set at 450nm and 550nm. Subtract 550nm values from 450nm values to correct for optical imperfections in the microplate. If an absorbance at 550nm is not available, measure the absorbance at 450nm only. When the 550nm measurement is omitted, absorbance values will be higher.



G. Calculation of Results

- The standard curve is used to determine rat IL-6 amount in an unknown sample. Generate the standard curve by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs. the corresponding rat IL-6 concentration (pg/mL) on the horizontal (X) axis.
- Calculate results using curve-fitting statistical software (linear or 4-parameter curve fit). Determine the rat IL-6 amount in each sample by interpolating from the absorbance value (Y-axis) to rat IL-6 concentration (X-axis) using the standard curve.
- Multiply the interpolated value by the dilution factor to calculate pg/mL of rat IL-6 in the sample.
- Absorbance values obtained for duplicates should be within 10% of the mean value. Carefully consider duplicate values that differ from the mean by greater than 10%.

Performance Characteristics

Sensitivity: < 16pg/mL
The sensitivity or lower limit of detection (LLD)[1] is determined by assaying replicates of zero and the standard curve. The mean signal of zero + 2 standard deviations read in dose from the standard curve is the LLD. This value is the smallest dose that is not zero with 95% confidence.

Assay range: 62.5-4000pg/mL
Suggested standard curve points are 4000, 2000, 1000, 500, 250, 125, 62.5 and 0pg/mL.

Reproducibility: Reproducibility of this assay is evaluated in each sample matrix. To determine the intra-assay precision, 10 replicates of samples containing two levels of recombinant rat IL-6 are assayed on a single plate (representative data shown). To evaluate inter-assay precision, samples are tested by three operators who perform separate assays. Ten replicates from three operators are used to calculate inter-assay precision data for each level of IL-6. Data are indicated in the table below:

		Intra-assay Precision			Inter-assay Precision		
Sample	Level	Mean (pg/mL)	SD (pg/mL)	CV (pg/mL)	Mean (pg/mL)	SD (pg/mL)	CV (pg/mL)
Serum	1	2858.4	176.8	6.2	3133.8	466.4	14.9
	2	922.9	62.7	6.8	1073.7	151.2	14.1
EDTA Plasma	1	3710.4	662.6	17.9	3499.5	793.3	22.7
	2	1235.6	66.0	5.3	1232.9	161.7	13.1
Citrate Plasma	1	3405.4	162.9	4.8	3080.0	321.9	10.5
	2	1310.2	82.1	6.3	1065.6	218.7	20.5
Heparin Plasma	1	2869.0	548.6	19.1	2511.5	531.9	21.2
	2	1086.6	137.1	12.6	930.7	165.4	17.8

Specificity: This ELISA is specific for the measurement of natural and recombinant rat IL-6. This ELISA does not cross-react (≥ 1%) with the following cytokines: rat IL-10 and TNFα; mouse IL-6, IL-1β, TNFα, IL-4, IL-2 and IL-1α; or human IL-8 and GM-CSF.

Calibration: The standard in this ELISA is calibrated to an internal rat IL-6 reference standard.

Expected values for supernatant from stimulated rat splenocytes: Rat spleens are harvested and splenocytes are isolated. Isolated splenocytes (6×10^6 cells/mL) are cultured with LPS (10µg/mL). Supernatants are collected at various time points and assayed for rat IL-6.

Spike and recovery: Recovery of rat IL-6 is evaluated using the Rat IL-6 ELISA Kit. Individual serum and plasma samples, diluted 1:3 in Standard Diluent, from Wistar rats and sample diluent controls are spiked with recombinant rat IL-6. Endogenous IL-6 levels are determined by evaluating non-spiked samples with spiked aliquots of the same samples. Expected values are calculated by adding endogenous IL-6 levels to those of spiked diluent controls. Percent (%) recovery is determined by dividing observed by expected values. Results for representative individual samples and populations are reported below.

		Representative Sample			Sample Population			
Sample	Level	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)	n
Serum	1	2855	2629	92	2862	2841	99	5
	2	1179	1094	93	1186	1075	91	5
EDTA Plasma	1	3003	2974	99	3003	3115	103	5
	2	1000	1002	100	1000	984	98	5
Citrate Plasma	1	2343	2351	100	2343	2412	102	5
	2	846	845	100	846	775	92	5
Heparin Plasma	1	2857	2786	98	2864	2704	94	5
	2	947	946	100	953	899	94	5

Dilution linearity: Individual serum and plasma samples diluted 1:3 in Standard Diluent from Wistar rats are spiked with recombinant rat IL-6, serially diluted in Sample Diluent, and evaluated in the ELISA. Dilution linearity for plasma is assessed for each anticoagulant. Representative data from each sample type are reported below. Observed values are compared to the expected values to calculate % recovery and demonstrate the assay's linearity of dilution.

Sample	Dilution	Expected (pg/mL)	Observed (pg/mL)	Recovery (%)
Serum	Neat	2185	2185	-
	1:2	1093	955	87
	1:4	546	493	90
	1:8	273	265	97
EDTA Plasma	Neat	2951	2951	-
	1:2	1476	1331	90
	1:4	738	730	99
	1:8	369	349	95
Heparin Plasma	Neat	2380	2380	-
	1:2	1190	1089	92
	1:4	595	617	104
	1:8	297	276	93
Citrate Plasma	Neat	2416	2416	-
	1:2	1208	994	82
	1:4	604	514	85
	1:8	302	242	80

Cited Reference

1. *Immunoassay: A Practical Guide,* Chan and Perlstein, Eds., 1987, Academic Press: New York, p71.

Data Templates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
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