Performance characteristics, continued

Intra-assay precision

Samples of known Rt TNF-α concentration were assayed in replicates of 16 to determine precision within an assay.

Parameters	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	130.7	241.8	912.1
SD	9.0	14.9	39.1
%CV	6.9	6.2	4.3

SD = Standard Deviation; CV = Coefficient of Variation

Recovery

The recoveries of Rt TNF-α added to rat serum, EDTA plasma, heparinized plasma, tissue culture media containing 1% fetal bovine serum, and tissue culture media containing 10% fetal bovine serum were measured with the Rt TNF-α ELISA Kit.

Sample	Range	Average % Recovery
Serum	83-97%	92
EDTA plasma	101-115%	106
Heparin plasma	106-122%	115
RPMI+1% fetal bovine serum	97-113%	105
RPMI+10% fetal bovine serum	108-110%	109

Expected values

Twenty rat serum and plasma samples were evaluated for detectable levels of Rt TNF-α in this assay. The mean value of the serum and plasma samples measured less than the lowest Rt TNF-α standard, 11.7 pg/mL. Rat splenocytes supernatants, from rats stimulated for 72 hours with 0.5 mg of LPS, cultured in the presence of 50 ng/mL PMA and 250 ng/mL calcium ionophore for 72 hours were assayed for Rt TNF-α and measured an average of 725 pg/mL.

High dose hook effect

No hook effect was observed with concentrations up to 1 µg/mL.

Inter-assay precision

Samples were assayed 36 times in multiple assays to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	135	259.2	970.9
SD	12.1	20.7	75.4
%CV	9.0	8.0	7.8

SD = Standard Deviation; CV = Coefficient of Variation

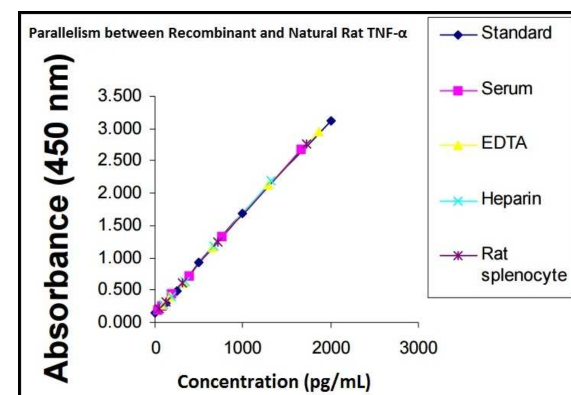
Linearity of dilution

Rat serum,EDTA plasma, and heparinized plasma samples (all spiked with natural Rt TNF-α) were serially diluted in Incubation Buffer, and supernatant from stimulated rat splenocytes was serially diluted in Standard Diluent Buffer over the range of the assay. Linear regression analysis of samples versus the expected concentration yielded the average correlation coefficients in the following table:

Sample	Correlation
Serum	0.997
EDTA plasma	0.974
Heparin plasma	0.988
Splenocyte supernatant	0.979

Parallelism

Supernatants from stimulated rat splenocytes were serially diluted in Standard Diluent Buffer, while rat serum and plasma samples spiked with natural Rt TNF-α were serially diluted in Incubation Buffer. The optical density of each dilution was plotted against the Rt TNF-α standard curve. Parallelism was demonstrated by the figure below and indicated that the standard accurately reflects the Rt TNF-α content in natural samples.



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Product label explanation of symbols and warnings

REF	Catalog Number	LOT	Batch code		Temperature limitation		Use by		Manufacturer		Consult instructions for use		Caution, consult accompanying documents

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Rat TNF-α ELISA Kit

Catalog nos. KRC3011 **Quantity:** 96 tests
KRC3012 192 tests
KRC3011C 480 tests

Pub. No. MAN0003966 **Rev** 1.0

Description

The Rat Tumor Necrosis Factor-Alpha (Rt TNF-α) ELISA Kit is a solid-phase sandwich Enzyme-Linked Immunosorbent Assay (ELISA). This assay is designed to detect and quantify the level of Rt TNF-α in rat serum, plasma, buffered solution, or cell culture medium. The assay will recognize both natural and recombinant rat TNF-α.

Contents and storage

The components included in the ELISA kit are listed below. Upon receipt, store the kit at 2°C to 8°C.

Components	Cat. No. KRC3011 96 tests	Cat. No. KRC3012 192 tests	Cat. No. KRC3011C 480 tests
Rt TNF-α Antibody Coated Wells. 96 well plate.	1 plate	2 plates	5 plates
Rt TNF-α Biotin Conjugate. Contains 0.1% sodium azide.	11 mL	22 mL	55 mL
Rt TNF-α Standard, recombinant Rt TNF-α. Contains 0.1% sodium azide. Refer to vial label for quantity and reconstitution volume.	2 vials	4 vials	10 vials
Wash Buffer Concentrate (25X).	100 mL	100 mL	200 mL
Incubation Buffer.	12 mL	24 mL	60 mL
Standard Diluent Buffer. Contains 0.1% sodium azide.	25 mL	50 mL	125 mL
Streptavidin-HRP (100X). Contains 3.3 mM thymol.	0.125 mL	0.250 mL	0.625 mL
Streptavidin-HRP Diluent. Contains 3.3 mM thymol.	25 mL	25 mL	75 mL
Stabilized Chromagen, Tetramethylbenzidine (TMB).	25 mL	25 mL	75 mL
Stop Solution.	25 mL	25 mL	75 mL
Adhesive Plate Covers.	3	6	15

CAUTION! This kit contains materials with small quantities of sodium azide. Sodium azide reacts with lead and copper plumbing to form explosive metal azides. Upon disposal, flush drains with a large volume of water to prevent azide accumulation. Avoid ingestion and contact with eyes, skin and mucous membranes. In case of contact, rinse affected area with plenty of water. Observe all federal, state, and local regulations for disposal.

Materials required but not provided

- Distilled or deionized water
- Microtiter plate reader with software capable of measurement at or near 450 nm
- Plate washer–automated or manual (squirt bottle, manifold dispenser, or equivalent)
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solutions

General Guidelines

- Review the **Procedural guidelines** and **Plate washing directions** in the *ELISA Technical Guide* available at **thermofisher.com/techresources** for details prior to starting the procedure.
- Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.

Prepare 1X Wash Buffer

1. Allow the Wash Buffer Concentrate (25X) to reach room temperature and mix to redissolve any precipitated salts.
2. Dilute 16 mL of Wash Buffer Concentrate (25X) with 384 mL of deionized or distilled water. Label as 1X Wash Buffer.
3. Store the concentrate and 1X Wash Buffer in the refrigerator. Use the diluted buffer within 14 days.

Sample preparation guidelines

- Collect samples in pyrogen/endotoxin-free tubes.
- Freeze samples after collection if samples will not be tested immediately. Avoid multiple freeze-thaw cycles of frozen samples. Thaw completely and mix well (do not vortex) prior to analysis.
- Avoid the use of hemolyzed or lipemic sera. If large amounts of particulate matter are present in the sample, centrifuge or filter sample prior to analysis.

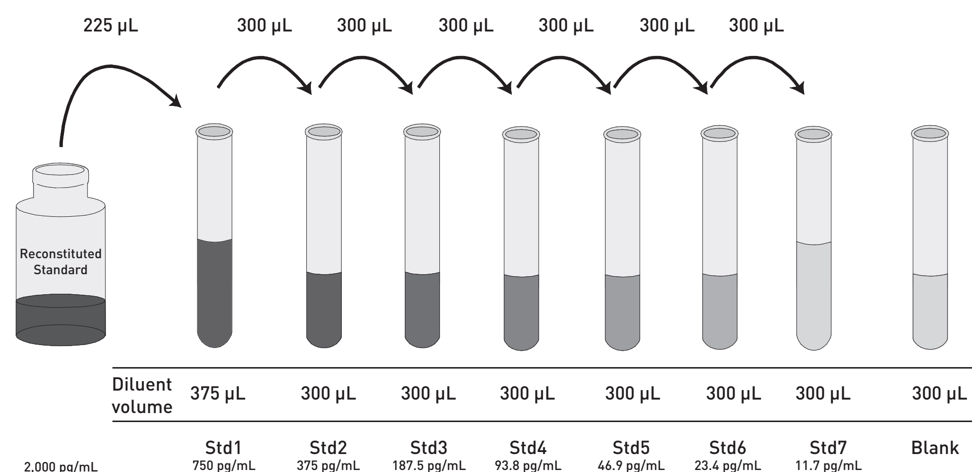
Dilute samples

- Dilute **serum and plasma** samples 2-fold in Incubation Buffer.
- Dilute **tissue culture supernatants** 2-fold in Standard Diluent Buffer.
- Because conditions may vary, it is recommended that each investigator determine the optimal dilution to be used for each application.

Dilute standards

Note: Use glass or plastic tubes for diluting standards.

1. Reconstitute Rt TNF-α Standard to 2,000 pg/mL with Standard Diluent Buffer. Refer to the standard vial label for instructions. Swirl or mix gently and allow the contents to sit for 10 minutes to ensure complete reconstitution. Label as 2,000 pg/mL Rt TNF-α.
   Use the standard within 1 hour of reconstitution.
2. Add 225 µL Reconstituted Standard to one tube containing 375 µL Standard Diluent Buffer and label as 750 pg/mL Rt TNF-α.
3. Add 300 µL Standard Diluent Buffer to each of 6 tubes labeled as follows: 375,187.5, 93.8, 46.9, 23.4, and 11.7 pg/mL Rt TNF-α.
4. Make serial dilutions of the standard as described below in the dilution diagram. Mix thoroughly between steps.
5. Discard any remaining reconstituted standard. Return the Standard Diluent Buffer to the refrigerator.



Prepare Streptavidin-HRP solution

Note: Prepare Streptavidin-HRP within 15 minutes of usage.

The Streptavidin-HRP (100X) is in 50% glycerol, which is viscous. To ensure accurate dilution:

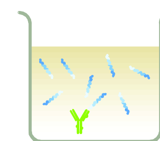
1. For each 8-well strip used in the assay, pipet 10 µL Streptavidin-HRP (100X) solution, wipe the pipette tip with a clean absorbent paper to remove any excess solution, and dispense the solution into a tube containing 1 mL of HRP Diluent. Mix thoroughly.
2. Return the unused Streptavidin-HRP (100X) solution to the refrigerator.

ELISA procedure

Allow all reagents to reach room temperature before use. Mix all liquid reagents prior to use. **Total assay time is 4 hours.**

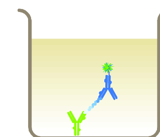
IMPORTANT! Perform a standard curve with each assay.

Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store at 2 to 8°C for future use.



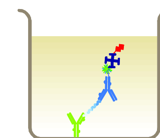
Bind antigen

1. Add 100 µL of standards, controls, or diluted samples (see page 2) to the appropriate wells.
2. Cover the plate with the plate cover and incubate for 2 hours at room temperature.
3. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.



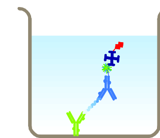
Add detector antibody

4. Add 100 µL Rt TNF-α Biotin Conjugate solution into each well except the chromogen blanks.
5. Cover the plate with plate cover and incubate for 1 hour at room temperature.
6. Thoroughly aspirate the solution and wash wells 4 times with 1X Wash Buffer.



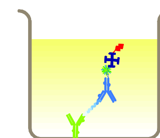
Add Streptavidin-HRP

7. Add 100 µL Streptavidin-HRP (see page 2) into each well except the chromogen blanks.
8. Cover the plate with the plate cover and incubate for 30 minutes at room temperature.
9. Thoroughly aspirate the solution from the wells and wash wells 4 times with 1X Wash Buffer.



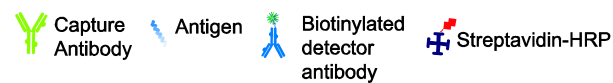
Add chromogen

10. Add 100 µL Stabilized Chromogen to each well. The substrate solution will begin to turn blue.
11. Cover the plate with the plate cover and incubate for 30 minutes at room temperature **in the dark**.
    Note: TMB should not touch aluminum foil or other metals.



Add stop solution

12. Add 100 µL of Stop Solution to each well. Tap side of the plate gently to mix. The solution in the wells changes from blue to yellow.



Read the plate and generate the standard curve

1. Read the absorbance at 450 nm. Read the plate within 30 minutes after adding the Stop Solution.
2. Use curve-fitting software to generate the standard curve. A four parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns and controls, prior to plotting.
3. Read the concentrations for unknown samples and controls from the standard curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.

   Note: Dilute samples producing signals greater than that of the highest standard in Standard Diluent Buffer and reanalyze. Multiply the concentration by the appropriate dilution factor.

Performance characteristics

Standard curve (example)

The following data were obtained for the various standards over the range of 0-750 pg/mL Rt TNF-α.

Standard Rt TNF-α (pg/mL)	Optical Density (450 nm)
750	3.29
375	1.93
187.5	1.03
93.8	0.59
46.9	0.32
23.4	0.18
11.7	0.13
0	0.05

Specificity

Buffered solutions of a panel of substances ranging in concentrations from 1.5 to 9.0 ng/ml were assayed with the Invitrogen Rt TNF-α ELISA Kit and found to have no cross-reactivity: **Rat** GM-CSF, IFN-γ, IL-1α, IL-1β, IL-1Ra, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12,, IL-13, MCP-1, MIP-1α, MIP-2, and RANTES; **human** TNF-α; and **swine** TNF-α. Recombinant **mouse** TNF-α protein demonstrated 100% crossreactivity with this kit.

Sensitivity

The minimum detectable dose of Rt TNF-α is <4 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero standard was assayed 30 times, and calculating the corresponding concentration.