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XpressBio
Life Science Products

Mouse TNF-α ELISA Assay
Catalog# CY-003

Introduction

Principle of the Assay

Microtitration wells coated with rat tumor necrosis factor alpha (TNF-α) capture antibody are exposed to test specimens, which may contain murine TNF-α cytokine. After an incubation period, unbound components in the test sample are washed away. Specifically bound TNF-α reacts with a mouse anti-TNF-α conjugated with biotin during a second incubation period. Following a second wash cycle the biotinylated antibody is detected by the addition of a streptavidin HRP conjugate. Following a third wash cycle, specifically bound enzyme conjugate is detected by reaction with the Substrate Solution, tetramethylbenzidine (TMB). The assay is measured spectrophotometrically to indicate the level of TNF-α present in a sample.

Kit Presentation

Materials Supplied
The reagents supplied in this pack are for Research use only.

		1 plate (96 wells)	2 plate (192 wells)
1	Coated microwell strips. Plastic microtitration wells coated with anti-mouse TNF-α rat monoclonal antibody in foil pouch with desiccant.	1 plate (96 wells)	2 plate (192 wells)
2	Positive mouse TNF-α calibrator (7000 pg/mL)	0.4 mL	2 x 0.4 mL
3	Detector antibody anti-mouse TNF-α conjugated to biotin.	12 mL	2 x 12 mL
4	Conjugate. Streptavidin conjugated to horseradish peroxidase enzyme containing 0.01% Bromonitrodioxane as preservative.	12 mL	2 x 12 mL
5	Wash Buffer (20x concentrated). Tris buffered saline pH 7.8-8.0, containing 0.05% Tween 20. Must be diluted before use.	1 x 60 mL	2 x 60 mL
6	Substrate Solution. Ready to use. Tetramethylbenzidine (TMB)	12 mL	2 x 12 mL
7	Stop Solution. 1 N H2SO4	12 mL	2 x 12 mL
8	Calibrator Diluent 1	6 mL	2 x 6 mL
9	Calibrator Diluent 2	6 mL	2 x 6 mL
10	Assay Diluent	30 mL	2 x 30 mL

Additional Requirements for Manual Processing
> Disposable tip micropipettes to deliver volumes of 5 μL, 10 μL, 25 μL, 100 μL and 200 μL (multichannel pipette preferred for dispensing reagents into microtiter plates).
> Distilled or deionized water.
> 37 (±1)º C incubator.
> Clean, disposable plastic/ glass test tubes, approximate capacities 5 mL and 10 mL.

> Range of standard, clean volumetric laboratory glassware consisting of, at least, 15 mL and 100 mL beakers, 1 L graduated cylinder,1 mL, 5 mL, and 10 mL glass pipettes.
> Absorbent paper towels.
> Automatic microtitration plate washer or laboratory wash bottle.
> Microtitration plate reader with 450 nm filter.
> Latex gloves, safety glasses and other appropriate protective garments.
> Biohazard infectious waste containers.
> Safety pipetting devices for 1 mL or larger pipettes.
> Timer.

Automatic, or Semi-automatic Processing

The mouse TNF-α Assay may be used with a variety of automatic or semi-automatic processors/liquid handling systems. It is essential that any such system is qualified, before it is used routinely, by demonstrating that the mouse TNF-α Assay results obtained using the automatic processor are equivalent to those obtained for the same specimens using the manual test method. Subsequently the automatic processor should be periodically re-qualified.

Storage and Stability

The positive control standard should be stored frozen at -70°C. It may be freeze thawed up to 5 times. All other reagents should be stored at 2-8°C, and should not be used beyond the expiration date on the label.   Once opened, microtitration strips may be stored at 2-8°C until the expiration date on the label, provided that desiccated conditions are maintained. Unused strips should be returned to their original foil pouch along with the sachet of desiccant. Opened pouches should be securely resealed by folding over the open end and securing it with adhesive tape.

The working strength Wash Buffer should not be stored for longer than 3 weeks at 2-8°C. It is recommended that Wash buffer be freshly diluted before each assay. If the working strength buffer becomes visibly cloudy or develops precipitate during the 3 weeks, do not use it.

Indications of Deterioration
The mouse TNF-α Assay may be considered to have deteriorated if:
1. The kit fails to meet the required criteria for a valid test (see interpretation of results).
2. Reagents becoming visibly cloudy or develop precipitate. *Note*: Concentrated Wash buffer, when cold, normally develops crystalline precipitates, which re-dissolve on heating at 37°C.
3. The Substrate Solution turns dark blue. This is likely to be caused by chemical contamination of the Substrate Solution.

Warnings and Precaution

Safety
1. The reagents supplied in this kit are for **Research use only**.
2. Caution: All blood products should be treated as potentially infectious. Essential precautions can be summarized as follows**:**
   >do not pipette by mouth.
   >Wear disposable gloves during all specimen and assay manipulations.
   >Avoid use of sharp or pointed liquid handling devices, which may puncture skin.
   >Do not smoke, eat or drink in the laboratory work area.
   >Avoid splashing of liquid specimens and reagents and the formation of aerosols.
   >Wash hands thoroughly on completion of a manipulation.
   >The Centers for Disease Control & Prevention and the National Institutes of Health recommend that potentially infectious agents be handled at the Biosafety Level 2.
3. The mouse TNF-α kits contain reagent systems, which are optimized and balanced for each kit lot. Do not interchange reagents from kits with different lot numbers. Do not interchange vial caps or stoppers either within or between kits.
4. The Substrate Solution and Stop Solution in this kit contain ingredients that can irritate the skin and cause eye damage. Handle them with care and wear suitable protective clothing and eye/face protection. In case of

contact with skin or eyes, immediately flush the affected area with plenty of water. For eyes, obtain medical attention.

Procedural

1. This kit should be used in strict accordance with the instructions in the Package Insert.
2. Do not use mouse TNF-α Assay kits after the expiration date printed on the outer carton label.
3. Do not cross contaminate reagents. Always use fresh pipette tips when drawing from stock reagent bottles.
4. Always use clean, preferably disposable, glassware for all reagent preparation.
5. Allow foil bags to warm to room temperature before opening. This avoids condensation on the inner surface of the bag, which may contribute to a deterioration of coated strips intended for future use.
6. Reagents should be dispensed with the tip of the micropipettes touching the side of the well at a point about mid-section. Follow manufacturer's recommendations for automatic processors.
7. Always keep the upper surface of the microtitration strips free from excess fluid droplets. Reagents and buffer over-spill should be blotted dry on completion of the manipulation.
8. Do not allow the wells to completely dry during an assay.
9. Disposal or decontamination of fluid in the waste reservoir from either the plate washer or trap for vacuum line in the manual system should be in accordance with guidelines set forth in the Department of Labor, Occupational Safety and Health Administration, occupational exposure to blood-borne pathogens; final rule (29 CFR 1910,1030) FEDERAL REGISTER, pp. 64176-84177,12/6/91.
10. Automatic or semi-automatic EIA processors or liquid handling systems should be qualified specifically for use with TNF-α Assay by demonstration of equivalence to the manual processing methods.
11. Consistent with good laboratory practice, it is recommended that all pipetting devices (manual or automatic), timers and thermometers are regularly calibrated according to the manufacturer's instructions.
12 Care must be taken to ensure that specimens are dispensed correctly to each test well. If a specimen is inadvertently not added to a well, the result for that well will be non-reactive, regardless of the actual result of the specimen.

Method of Use

Specimen Collection and Storage
The mouse TNF-α Assay is intended for use with blood serum and tissue culture supernatants. The specimen should be tested as soon as possible. However, if the specimen needs storage, the specimens should be stored frozen at -20°C or below. Do not use self-defrosting freezers. Specimens that have been frozen and thawed should be thoroughly mixed before testing.

Rinse Cycle
Efficient rinsing to remove un-complexed components is a fundamental requirement of enzyme immunoassay procedures. The TNF-α assay utilises three standard six-rinse cycles. Automatic plate washers may be used provided they meet the following criteria:
1 All wells are completely aspirated.
2. All wells are filled to the rim (350 μL) during the rinse cycle.
3. Wash buffer is dispensed at a good flow rate.
4. The microtitration plate washer must be well maintained to prevent contamination from previous use. Manufacturer's cleaning procedures must be followed diligently

For each rinse cycle the machine should be set to six consecutive washes. On completion of the cycle, invert the microtitration plate and tap firmly on absorbent paper towels. Check for any residual Wash buffer in the wells and blot dry the upper surface of the wells with a paper towel.

Alternatively, the following manual system may be employed:
1. Aspirate well contents using a vacuum line fitted with a trap.
2. Fill all wells to the brim with Wash buffer dispensed from a squeeze-type laboratory wash bottle.
3. Aspirate all wells.
4. Repeat steps 2 and 3, five additional times.
5. Invert the microtitration plate and tap firmly on absorbent paper towels.

Preparation for the Assay

1.   Positive Mouse TNF-α Calibrator 7000 pg/mL
a. Prepare working strength calibrator by diluting 50ul of the positive mouse TNF-α calibrator into 450 uL (1:10 dilution) of the respective calibrator diluent (see NOTE below). This will give a final concentration of 700 pg/mL and will be used at the top standard. **NOTE: When assaying cell culture samples, dilute positive mouse TNF-α calibrator using calibrator diluent 1. When assaying blood serum samples, dilute mouse TNF-α calibrator using calibrator diluent 2**.

b. Prepare six serial two-fold dilutions (200ul standard with 200ul of calibrator diluent) starting with the 700 pg/mL top standard to prepare 350 pg/mL, 175 pg/mL, 87.5 pg/mL, 43.8 pg/mL, 21.9 pg/mL, and 10.9 pg/mL calibrators. Each calibrator plus a zero standard (0 pg/mL, calibrator diluent only) should be run in duplicate.

2.   Wash Buffer
Prepare working-strength Wash buffer by diluting 1-part concentrate with 19-parts of distilled or de-ionized water. If a kit is likely to be utilized over a period in excess of 4 weeks, then it is recommended that only enough stock concentrate be diluted sufficient for immediate needs. Each row of 8 wells may be adequately washed with 150 mL of working strength buffer.

Assay Procedure
1 Allow all reagents to reach room temperature (18-25°C).
2 Select sufficient microtitration well strips to accommodate all test specimens, calibrators and zero standard. Fit the strips into the holding frame. Label wells according to specimen identity using the letter/number cross-reference system molded into the plastic frame.
3 Add 50 μL of assay diluent to each well.
4 Dispense 50 μL of each calibrator and specimen into appropriate wells. **NOTE: All calibrators and samples should be tested in duplicate.**
5 Incubate at 37(±1)°C for 60 (±5) minutes.
6 Aspirate the contents of the wells and wash the microtitration plate as described in the Rinse Cycle section.
7 Pipette 100 μL of detector antibody into each well and incubate at 37(±1)°C for 60 (±5) minutes.
8 Aspirate the detector antibody from the wells and wash the microtitration plate as described in the rinse cycle section.
9 Pipette 100 μl of Streptavidin HRP conjugate into each well and incubate at room temperature (18-25°C) for 30 (±5) minutes.
10 Aspirate the conjugate from the wells and wash the microtitration plate as described in the Rinse Cycle section.
11 Without delay, dispense 100 μL Substrate Solution (TMB) into each well. A multichannel pipette should be used for best results. Leave at room temperature (18-25°C) protected from direct sunlight, for 30 (±2) minutes.
12 Stop the reaction by adding 100 μL of Stop Solution to each well including the reagent blank. The blue solution should change to a uniform yellow color. Ensure that the undersides of the wells are dry and that there are no air bubbles in the well contents.
13 Immediately after adding the Stop solution, read the absorbance values at 450 nm using a microtitration plate reader blanked on the negative control well.

Interpretation of Results

The following criteria should be met for a valid assay:

For Cell Culture Standard Curve:
The zero standard should be ≤ 0.10
The 350 pg/mL control should be ≥0.60

For Mouse Serum Standard Curve:
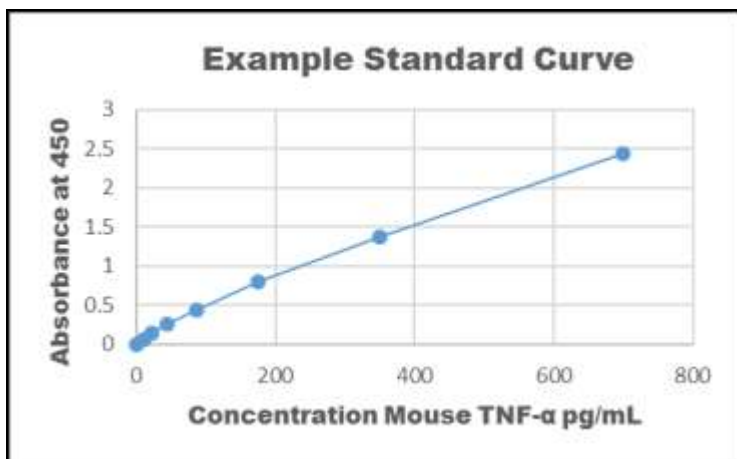The zero standard should be ≤ 0.10
The 350 pg/mL control should be ≥0.40

Quantitative Analysis

Manual Method: The calibration curve can be constructed manually on linear graph paper by plotting the mean absorbance for each standard on the y-axis versus the concentration of the standard (value printed on vial) on the x-axis. Connect the points to produce a point to point curve. Do not force the line to be linear. The concentration of the specimens can be found directly from the standard curve

Table 1. Example Data at 450 nm.

Sample	450 nm abs.	
Standard 1 (0 pg/mL)	0	
"		
Standard 2 (10.9 pg/mL)	0.055	
"		
Standard 3 (21.9 pg/mL)	0.135	
"		
Standard 4 (43.8 pg/mL)	0.25	
"		
Standard 5 (87.5 pg/mL)	0.436	
"		
Standard 6 (175 pg/mL)	0.793	
"		
Standard 7 (350 pg/mL)	1.379	
"		
Standard 8 (700 pg/mL)	2.44	
		pg/mL
Specimen #1	2.143	590
"		
Specimen #2	2.189	603
"		

Computer-Assisted Method: Computer assisted data reduction may be used to create the standard curve. Software providing a point to point curve fitting routine provides acceptable results.

Procedure for samples with mouse TNF-α assay values greater than the highest standard.

Many tissue culture samples will have TNF-α values greater than the highest standard. In order to obtain accurate results for samples with mouse TNF-α assay values greater than the highest standard it is necessary to dilute and re-test the sample. Diluting the serum specimen 10-fold is recommended. For example: Make a 10-fold dilution by adding 0.10 mL of the initial specimen to 0.90 mL of tissue culture medium. Mix thoroughly and repeat the assay according to the Assay Procedure. Multiply the results by 10 to determine the correct TNF-α assay values in the sample.

Limitations of Use

1. Assay values determined using assays from different manufacturers or different methods may not be used interchangeably.
2. The assay cannot be used to quantitate samples with mouse TNF-α assay values greater than the highest standard without further serial dilution of the samples. See the Interpretation of Results section for directions on testing such samples.
3. The performance characteristics have not been established for any matrices other than tissue culture media and mouse serum.

Note: This standard curve is only an example and should not be used to generate any results.

Performance Characteristics

Analytical Sensitivity: To determine the sensitivity of the assay, the zero standard was assayed 20 times. The minimal detectable level was calculated by adding two standard deviations to the mean absorbance for the 0 standard. The minimal detectable level for TNF-α was 3.63 pg/mL.

Linearity: Four strongly reactive samples were serially two fold diluted and run on the assay. The values obtained were compared to the expected values by standard linear regression. The r values obtained ranged from 0.997 to 1 with an average of 1.

Recovery: Mouse TNF-α recovery was tested in both cell culture media and mouse serum. Recovery for cell culture media was 91% with a range of 83-98%. Recovery for mouse serum was 85% with a range of 80-90%

Calibration: Mouse TNF-α was calibrated using NIBSC/WHO 88/532 standard and measured as follows:

NIBSC/WHO 88/532: 1 Unit of standard = 2.87 pg/mL mouse TNF-α

Precision: Four samples with different levels of activity were assayed ten times each on three different assays. The results are summarized in the following table.

Precision Data

		Sample 1	Sample 2	Sample 3	Sample 4
Assay 1 (n = 10)	Mean (pg/mL)	455.92	217.41	95.90	44.40
	SD	25.69	12.59	6.62	3.93
	CV	5.63%	5.79%	6.90%	8.84%
Assay 2 (n = 10)	Mean (pg/mL)	456.05	221.29	99.33	45.88
	SD	26.43	11.37	6.58	5.86
	CV	5.80%	5.14%	6.63%	12.76%
Assay 3 (n = 10)	Mean (pg/mL)	459.54	236.51	109.26	52.10
	SD	15.86	8.10	3.65	4.24
	CV	3.45%	3.42%	3.34%	8.14%
Inter-Assay (n = 30)	Mean (pg/mL)	457.17	225.07	101.50	47.46
	SD	22.42	13.41	8.02	5.70
	CV	4.90%	5.96%	7.90%	12.02%

Expected Values

Serum - Twenty individual mouse serum samples were analyzed for the presence of mouse TNF-α. All 20 samples measured less than the low standard of 10.9 pg/mL.

Cell Culture Supernatants - Mouse primary bone marrow-derived macrophages (BMM) were cultured for 6 days in DMEM supplemented with 10% fetal calf serum, 2 mM L-Glutamine and 10 ng/mL Macrophage Colony-Stimulating Factor. BMM were then treated for 24 hours with 100 ng/mL lipopolysaccharide. The cell culture supernatant produced 592 pg/mL of mouse TNF-α. The same samples produced 567 pg/mL with a competitor mouse TNF-α ELISA assay.

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